

Transforming growth factor β superfamily signaling and its role in B-cell lymphoma

by

Maren Bakkebø

Department of Immunology
Institute for Cancer Research
The Norwegian Radium Hospital
Oslo University Hospital

Centre for Cancer Biomedicine
Faculty of Medicine
University of Oslo



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Errata

The following corrections (underlined) have been made in the text:

- Page 7, paragraph 1, line 1: ABC Activated B-cell like
- Page 7, paragraph 1, line 23: GCB Germinal center B-cell like
- Page 11, paragraph 1, line 10: ...through SHM and Ig class switch...
- Page 15, paragraph 2, line 3: ...germinal_center B-cell like (GCB), activated B-cell like (ABC)...
- Page 16, paragraph 2, line 1: Burkitt's lymphoma (BL) can...
- Page 19, paragraph 2, line 8: ...carboxy-terminal ends...
- Page 20, paragraph 2, line 7: ...T β RI phosphorylation...
- Page 24, paragraph 3, line 8: ...Smad_independent.
- Page 32, paragraph 1, line 7: ..."stem-cell_properties".
- Page 47, paragraph 2, line 1: ...detected constitutive active ERK1/2 MAPK...
- Page 49, paragraph 2, line 10: ...TAg cells...
- Page 51, paragraph 1, line 8: ... gross enlargement of endosomes.
- Page 53, paragraph 1, line 7: ...mislocalization of...

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Abbreviations

ABC	Activated B-cell like
ActR	Activin type receptor
Alk	Activin receptor-like kinase
ATF3	Activating transcription factor 3
BCL	B-cell lymphoma
BCR	B-cell receptor
BL	Burkitt's lymphoma
BMP	Bone morphogenetic protein
BMPR	BMP type receptor
CDK	Cyclin-dependent kinase
Co-Smad	Common Smad
cPML	Cytoplasmic promyelocytic leukaemia
CSR	Class switch recombination
D	Diversity
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ESCRT	Endosomal sorting complex required for transport
FL	Follicular lymphoma
GC	Germinal center
GCB	Germinal center B-cell like
GS	Glycine-serine
HLH	Helix-loop-helix
ID	Inhibitor of differentiation
Ig	Immunoglobulin
IgH	Ig heavy chain
I-Smad	Inhibitory Smad
J	Joining
LMP-1	Latent membrane protein 1
MAPK	Mitogen-activated protein kinase
miRNA/miR	MicroRNA
NHL	Non-Hodgkin lymphoma
NSCLC	Non-small cell lung carcinoma
R-Smad	Receptor-regulated Smad
SARA	Smad anchor for receptor activation
SHM	Somatic hypermutation
T β R	TGF- β receptor
T _{FH}	Follicular T helper cell
TGF	Transforming growth factor
TGIF	TG-interaction factor
TRAF6	Tumor necrosis factor receptor-associated factor 6
V	Variable

List of included papers

- I. Kanutte Huse, **Maren Bakkebo**, Morten P. Oksvold, Sébastien Wälchli, Vera I. Hilden, Lise Forfang, May L. Bredahl, Knut Liestøl, Ash A. Alizadeh, Erlend B. Smeland and June H. Myklebust. Mechanistic basis for resistance to BMP-induced growth inhibition in B-cell lymphoma. (submitted)
- II. **Maren Bakkebo**, Kanutte Huse, Vera I. Hilden, Erlend B. Smeland and Morten P. Oksvold. TGF- β -induced growth inhibition in B-cell lymphoma correlates with Smad1/5 signalling and constitutively active p38 MAPK. BMC Immunology 2010. 11: 57.
- III. **Maren Bakkebo**, Nina Marie Pedersen, Marina Vietri, Lise Forfang, Vera I. Hilden, Knut Liestøl, Sebastian Patzke, June H. Myklebust, Harald Stenmark, Erlend B. Smeland and Morten P. Oksvold. SARA has a role in EGF receptor trafficking, but is not essential in TGF- β signaling. (submitted)

1. Introduction

1.1 The immune system

The immune system has developed to protect the host against pathogens like bacteria, viruses and parasites. It is a system comprised of both cellular and humoral components, which are interdependable to fight the invading organisms. An important feature is the possibility to discriminate between self and foreign, which is the reason why the immune system normally does not attack the body's own proteins or polysaccharides. The immune system in vertebrates consists of an innate and an adaptive part [1]. The innate immune system is a primary defense mechanism, acting less specific but faster than the adaptive immune system. Cells of the innate immune system, e.g. macrophages and granulocytes, recognize pathogens via pattern recognition receptors, binding to pathogen-associated molecular patterns. Among these receptors the Toll-like receptors are of high importance [2], binding e.g. lipopolysaccharides (LPS). These receptors are germline-encoded, which results in less divergence among the pattern recognition receptors compared to the receptors of the adaptive immune system. The innate immune system is essential in activating and instructing the adaptive immune response.

The adaptive immune system targets the pathogens broader and more flexible due to its high-affinity receptors, which are tailor-made for each antigen through recombination and somatic hypermutation (SHM) [3]. Another important feature of the adaptive immune system is the development of memory cells that persist in the body and facilitate a quick response to re-infections [4;5]. However, the adaptive immunity needs time to develop and it needs to be activated and directed by the innate immune system [6]. The cellular components of the adaptive immune system are T cells and B cells. T cells can further be divided into CD4⁺ helper T cells and CD8⁺ cytotoxic T cells [3]. The helper T cells are needed to support and activate B cells when they have encountered their antigen.

1.1.1 B cells

B cells have several roles in the immune system. They are antigen-presenting cells, presenting antigens to T cells, but their main function is to produce immunoglobulins (Igs), the humoral

component of the adaptive immune system. Different B-cell subtypes have been detected. The B-1 B lymphocytes account for ca. 5% of the B cells and are localized to serous cavities, and the B-2 marginal zone B lymphocytes are localized in the spleen. However, in the scope of this thesis the focus will be on the B-2 follicular B lymphocytes, the major group of the B cells [7].

1.1.1.1 *B-cell development: From stem cell to naive B cell*

The B-cell development in adults starts in the bone marrow, where a hematopoietic stem cell differentiates into early lymphoid progenitor cells, which can develop into immature B cells through distinct well-characterized steps [7;8]. From early lymphoid progenitor onwards through the pre-B cell stage the gene rearrangements occur to successfully produce an Ig heavy chain (IgH). The genes encoding for the heavy chain consists of three regions, the variable (V), diversity (D) and joining (J) regions with different gene segments. The V-D-J segments in the genome need to be recombined before the pre-B cell can express the pre-B-cell receptor (pre-BCR) on its surface. Signaling through the pre-BCR, which consists of the rearranged μ heavy chain and the surrogate light-chain proteins VpreB and $\lambda 5$, induces gene expression that allows the pre-B cells to further differentiate. This process is known to be antigen-independent. Next, the B-cell receptor (BCR) light chain is recombined. The expression of a functional BCR together with Ig α and Ig β , which are the signaling proteins of the BCR complex, is a prerequisite for the B cells to leave the bone marrow. When the B cells are released into the blood stream they are called immature B cells. They have Ig of type M and later also type D expressed on their cell surfaces. The immature B cells traffic to the spleen, where they develop further into naive B cells. During the developmental process from immature B cells to naive B cells the negative selection of self-reactive B cells occurs. In this process, B cells that bind to a self protein with their BCR and create a sufficient signal will undergo apoptosis. This diminishes the risk for autoimmunity.

1.1.1.2 *B-cell antigen encounter*

The naive B cells circulate the body via blood and lymph vessels. They survey secondary lymphatic tissues, e.g. lymph nodes, and the periphery on the search for their antigen. Upon antigen encounter in the periphery, the B cells move to the nearest lymph node [9;10]. The cell types in the lymph node are enriched in different areas, e.g. B cells and T cells are located in their

respective B-cell zones and T-cell zones. After antigen-binding the B cell surveys the T cells at the border between the T-cell zone and the B-cell zone, searching for a T cell that has high affinity for the same antigen. Only this T cell is able to activate the B cell. Once a B cell has encountered its corresponding T cell, they communicate via surface molecules, e.g. CD40 (B cell) and CD40L/CD154 (T cell), and different cytokines. Many of the proteins involved belong to the tumor necrosis factor (TNF) or tumor necrosis factor receptor (TNFR) superfamily. After activation, the B cell has two potential fates: 1) It migrates to extrafollicular areas in the lymph node where it proliferates and differentiates into transient plasmablasts and later plasma cells, which are both Ig-secreting cells, or 2) The activation of a B cell leads to formation of germinal centers (GCs) [9]. The GC is the site where B cells proliferate, go through SHM and Ig class switch recombination (CSR) and mature to memory B cells or plasma cells (Fig. 1). These plasma cells secrete Igs with higher affinity than the initial transient plasmablasts and plasma cells.

1.1.1.3 *Germinal centers*

A mature GC is constituted of a dark zone and a light zone [9;10], and the B cells can migrate between these zones. The dark zone is situated close to the T-cell zone, and harbors proliferating B cells without Ig cell-surface expression, termed centroblasts. The light zone consists of a network of follicular dendritic cells, follicular helper T cells (T_{FH}) and B cells, which no longer are mitotic and express Igs on their surface, called centrocytes (Fig. 1). The transcription factor B-cell lymphoma (BCL) 6 is upregulated in GC B cells and also in T_{FH} cells, and is known as the master regulator of the GC reaction [11;12]. BCL6 negatively regulates the transcription of target genes involved in apoptosis and cell-cycle control and further differentiation of the B cells. In this way, BCL6 hinders the differentiation of a GC B cell into a plasma cell until the Ig has completed class switch, affinity maturation and has been found not to be autoreactive.

During the GC reaction, specific regions of the rearranged BCR locus undergo SHM and CSR. In the process of SHM, point mutations occur on rearranged genes encoding primarily the variable region of the heavy and light chains [13], and during CSR the heavy chain of the BCR is substituted to either γ , α or ϵ [14]. For both processes the enzyme activation-induced deaminase

(AID) is essential. The centrocytes' Ig-affinity to their specific antigen increases dramatically during the GC reaction. In the light zone, the affinity of the Igs is tested, and B cells that fail to produce a proper Ig or which Igs' affinity is not sufficiently high, undergo apoptosis.

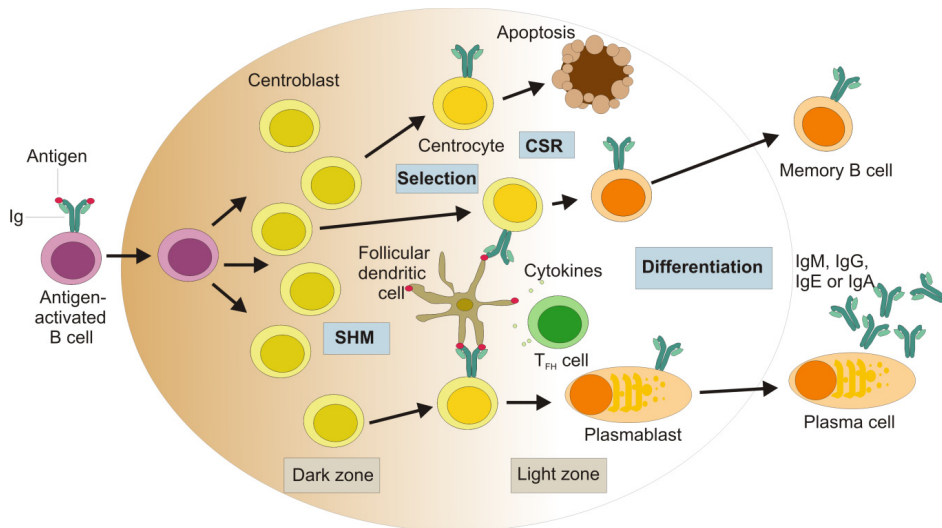


Figure 1. B-cell differentiation in the germinal center

Antigen-activated B cells enter the GC in the dark zone, where they start to proliferate and undergo somatic hypermutation (SHM). In this state the B cells are termed centroblasts. Later they migrate into the light zone for selection by follicular dendritic cells, with help from follicular helper T (T_{FH}) cells, and are now termed centrocytes. In the light zone cells can undergo class switch recombination (CSR). The centrocytes can cycle back to the dark zone for further rounds of proliferation. Centrocytes are either neglected and undergo apoptosis, or they are selected to become memory B cells or plasmablasts/plasma cells. The B-cell fate depends on the affinity of their receptor. After differentiation to either memory B cells or plasma cells the B cells leave the GC. (Modified from Klein and Dalla-Favera, 2008 [10]).

1.2 Lymphoma

A number of processes are common to cancer development in most cell types [15]. These hallmarks of cancer are sustained proliferative signaling, evasion of growth suppressors, invasion and metastasis, replicative immortality, induction of angiogenesis and resistance to cell death. The hallmarks are acquired via distinct mechanisms in different cancer types. However, what makes it possible is the genomic instability in the cancerous cells that might cause mutations, and the inflammatory state created by cells of the immune system, which can be tumor promoting. Lymphoma is a neoplastic malignancy in lymphocytes, which presents as a solid tumor in a lymph node [16]. Lymphomas are classified as Hodgkin's or Non-Hodgkin lymphoma (NHL), where NHL represents the largest group. There are several subtypes of both Hodgkin's lymphoma and NHL. Lymphoma can develop from B cells, T cells and natural killer (NK) cells. B-cell lymphomas are more common, accounting for about 95% of the incidences [17].

1.2.1 Non-Hodgkin B-cell lymphomagenesis

A hallmark of many B-cell lymphoma subtypes is the translocation of an oncogene, where the oncogene is subjected to the control of the Ig promoter. This leaves the oncogene constitutively expressed [17;18]. During V_H - D_H - J_H -recombination in the bone marrow and, in addition, during SHM and CSR in the lymph nodes, mutations in non-Ig genes may also arise. The location of the breakpoint indicates during which process the translocations have occurred. First, breakpoints directly adjacent to the J_H gene segments or adjacent to the D_H - J_H joining, are typical mutations from the V_H - D_H - J_H -recombination, e.g. the BCL2 t(14;18) translocation in follicular lymphoma (FL). Second, breakpoints found adjacent to or within the rearranged VDJ genes, which are somatically mutated, are typical of SHM. Third, breakpoints found in the IgH constant switch regions have typically occurred during CSR, often found in certain diffuse large B-cell lymphomas (DLBCLs) which overexpress activation-induced deaminase (AID) (Fig. 2). These single mutations are considered to be the initial event, but they are not sufficient for lymphoma to develop as some of the known lymphoma translocations and mutations, e.g. BCL2 and BCL6, are also found in healthy individuals [19;20]. Translocations not involving the Ig loci or mutations of other genes, e.g. c-Myc and Pim1, have also been reported in NHL [17;18]. In addition, viruses can be the cause of translocations in certain NHL subtypes.

Whereas lymphomas previously were thought to be autonomous, recent investigations have unraveled the role of the surrounding microenvironment in promoting survival of the lymphoma cells [21]. Many lymphomas, as their normal counterparts, are dependent on key survival factors and signaling through their BCR to survive [17].

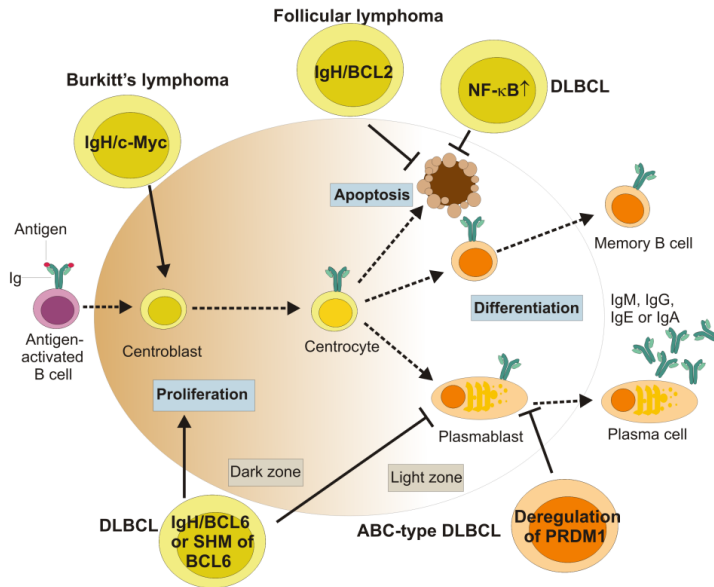


Figure 2. Molecular mechanisms of lymphomagenesis

Mistakes can occur at several steps during B-cell development. In general, the molecular mechanisms behind either promote proliferation or inhibit apoptosis or differentiation. Overexpression of c-Myc or BCL6 leads to enhanced proliferation. Upregulation of BCL2 or nuclear factor κB (NF-κB) can block apoptosis and mutations in BCL6 or deregulation of positive-regulatory-domain-containing 1 (PRDM1) can block differentiation. (Modified from Klein and Dalla-Favera, 2008 [10]).

1.2.2 B-cell lymphoma subtypes

Several B-cell lymphoma subtypes have been described as new tools for classification have been developed [16]. The different lymphoma subtypes are defined by morphologic, immunophenotypic, genetic and clinical features. Many different subtypes of lymphoma can develop. The different lymphoma subtypes can be seen as frozen stages in the development of a naive B cell [17]. Most tumors are classified according to their normal counterparts; however, this has proven difficult for some lymphoma subtypes, as their normal counterparts are not found. The new classification of lymphoma subtypes has revealed distinct prognostic outcomes, thus, it harbors the potential of a more suited therapy regime for each subtype [22].

1.2.2.1 *Diffuse large B-cell lymphoma*

DLBCL is the most common NHL in the Western world, accounting for 30-40% of new lymphoma cases [18]. It is an aggressive type of lymphoma. DLBCL can further be divided into the subtypes germinal center B-cell like (GCB), activated B-cell like (ABC) and, less frequent, primary mediastinal B-cell lymphoma, as identified by gene expression profiling [18;23;24]. They differ highly in their genetic abnormalities and arise from distinct stages in the B-cell differentiation. However, deregulation of BCL6 expression is found in all subtypes, although more frequently in the ABC subtype. BCL6 is the main regulator of GC B-cell differentiation, and deregulation of its expression may facilitate lymphomagenesis [12]. In GCB DLBCL, the cells still undergo SHM, and typically have completed CSR, thus, they resemble the GC B cell [18;23]. The BCL2 t(14;18) translocation and deletion of the tumor suppressor phosphatase and tensin homolog (PTEN) are other classical molecular features. In ABC DLBCL, the cells resemble plasmablasts, e.g. through expression of the X-box binding protein 1 (XBP1); however, they have not completed class switch [18]. Constitutive activation of the nuclear factor κ B (NF- κ B), overexpression of BCL2 or deletions of loci for p16 and p53, typical tumor suppressors, are common for many ABC DLBCLs [18;23]. In a fraction of ABC DLBCL, positive-regulatory-domain-containing 1 (PRDM1), the gene coding for the protein B-lymphocyte-induced maturation protein 1 (BLIMP1) is inactivated [10]. This protein is important for the differentiation of GC B cells.

1.2.2.2 *Follicular lymphoma*

FL represents the second most common lymphoid tumor and accounts for about 20% of the B-cell lymphoma cases [25]. The tumor consists of a mixture of neoplastic centroblasts and centrocytes, resembling a GC. The typical molecular feature in FL is the t(14;18) translocation, where BCL2 is regulated by the IgH-promoter; blocking apoptosis in these cells. However, this is not sufficient to develop FL, as the translocation also can be found in healthy individuals [19]. FL is an indolent lymphoma, but can transform into the more aggressive DLBCL.

1.2.2.3 *Burkitt's lymphoma*

Burkitt's lymphoma (BL) can be divided into Epstein-Barr virus (EBV)-positive or -negative BL. EBV-positive BL is more common in equatorial Africa [26]. EBV-positive BL has also been referred to as endemic BL, as opposed to sporadic or immunodeficient BL, although sporadic BL also can be EBV-positive [27]. Immunodeficient BL is often associated with human immunodeficiency virus (HIV) infection. EBV-positive BL has also been associated with malaria infection, and one theory is that a dysregulation of the immune system leads to B cells that are more prone to infection by EBV. BL is an aggressive lymphoma, more commonly present in children and young adults. The c-Myc t(8;14) translocation and constitutive activation is present in both EBV-positive and -negative BL [26;27].

1.3 The transforming growth factor β superfamily of cytokines

The transforming growth factor β (TGF- β) superfamily of cytokines is a large family comprised, in humans, of at least 30 related polypeptides, including TGF- β s, activins/inhibins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) (Fig. 3) [28]. A common structural motif in these cytokines is the presence of a cysteine knot formed by disulphide bonds between six conserved cysteine residues. Typically the mature protein presents as a dimer with intermolecular disulphide bonds.

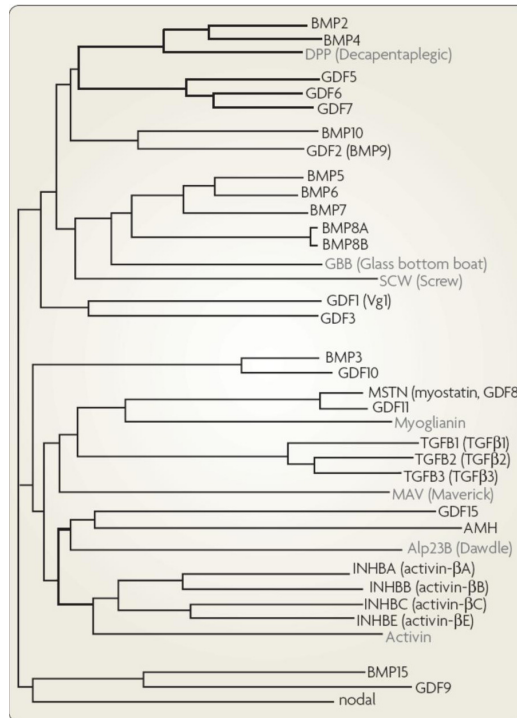


Figure 3. Phylogenetic tree of the TGF- β superfamily of cytokines

This figure shows a phylogenetic tree of the members of the TGF- β superfamily of cytokines in mammals (black) and *Drosophila* (grey). Subfamilies like the BMP family or activin family can be discriminated from the TGF- β family. (Modified from Schmierer and Hill, 2007 [28]).

All members of the TGF- β superfamily are highly conserved during evolution, and exert their many effects both during embryogenesis and in adult tissue homeostasis [29-31]. Some of the effects include regulation of cell growth, adhesion, migration, apoptosis and differentiation. TGF- β is one of the most potent growth-inhibitory morphogens in humans, inducing cell-cycle arrest or apoptosis in several different cell types. Members of the TGF- β superfamily are expressed in most cell types [32]. In addition, TGF- β superfamily receptors and intracellular signaling proteins are widely expressed, demonstrating the importance of this cytokine superfamily.

1.3.1 TGF- β

Three different TGF- β s are known in mammals, TGF- β 1, 2 and 3 [28]. Among those, TGF- β 1 is the most common cytokine, and will be the focus of this thesis, referred to as TGF- β . The TGFs were discovered in 1978, when growth factors, called sarcoma growth factors, were isolated from murine sarcoma virus-transformed cells [33], and later, in 1980, the TGFs were defined as a class of proteins with similar properties [34]. The name TGF originated from the discovery that the cytokine was produced by transformed cells and could induce transformation of untransformed cells by inducing soft agar colony growth. Anzano and colleagues later isolated both transforming growth factor α (TGF- α) and TGF- β from conditioned medium from sarcoma virus-transformed cells [35].

TGF- β is secreted as a dimer and as part of a large latent complex, which normally interacts with the extracellular matrix. In this complex, TGF- β is non-covalently attached to its pro-peptide, the latency-associated protein (LAP), which again is attached to latent TGF- β -binding protein [36-38]. This renders TGF- β biologically inactive. BMP-1, a proteinase, has been proposed to cleave off the latent TGF- β -binding protein part of the complex [39]. In addition, the latency-associated protein (LAP) needs to be cleaved off, and several mechanisms have been proposed for this cleavage, e.g. different matrix metalloproteinases (MMPs) [40;41].

1.3.1.1 *Receptor binding*

In humans, there are in total five type II receptors and seven type I receptors (the type I receptors are also referred to as activin receptor-like kinase (Alk) 1 to 7) facilitating signaling by the TGF- β superfamily [28]. TGF- β only binds to the TGF- β receptor II (T β RII) and Alk-5 (T β RI) or Alk-1. Alk-1 is primarily expressed on endothelial cells [42;43], thus, Alk-5 represents the most common TGF- β type I receptor. TGF- β induces signaling from a heterotetrameric receptor complex [44], comprised of two T β RIIs and two T β RIIs. TGF- β binds to the type II receptor, which then recruits the type I receptor and signaling can be induced [44].

Internalization of the T β Rs occurs independently of ligand binding [45;46]. However, there are contradictions in the findings regarding whether the signaling is induced from the cell surface [47], or whether the whole complex is internalized via clathrin-coated pits to early endosomes and generates signaling from there [48;49]. Most likely, signaling can be induced both from the cell surface and from endosomes. It is unclear whether both of these signaling modes are essential or not. Caveolin-1-mediated and lipid-raft-mediated endocytosis has been proposed to be involved in receptor degradation [49]. In addition to the main receptors, several co-receptors are known, which either facilitate or inhibit signaling [28;32]. Known TGF- β co-receptors are betaglycan (T β RIII) and endoglin. Both have been demonstrated to bind TGF- β , but there is no signaling emerging from these co-receptors. Nevertheless, the binding to co-receptors facilitates the binding to the T β Rs, thus the co-receptors promote signaling in an indirect fashion.

1.3.1.2 *Smad proteins*

The Smads are the intracellular signaling proteins common to the TGF- β superfamily of cytokines. Several different Smads exist and they can be divided into three groups [28;32;50]. The receptor-regulated Smads (R-Smads) comprise the largest group, with Smad1, 2, 3, 5 and 8. The common Smad (Co-Smad), Smad4, is shared by all members of the TGF- β superfamily. The last group is the inhibitory Smads (I-Smads), which consists of Smad6 and 7. The R-Smads and Co-Smad are proteins with high homology, and they are highly conserved throughout species. They are constituted of three domains, the MH1- and MH2-domains, situated at the amino- and carboxy-terminal ends, respectively, and the less homolog linker region, which links MH1 to MH2. The unphosphorylated Smad proteins constitutively translocate between the cytoplasm and the nucleus [51;52]. The MH2-domain in R-Smads mediates the Smad-receptor interaction and comprises the site for the receptor phosphorylation. Receptor-mediated phosphorylation occurs on the carboxy-terminal SSXS-motif where the serine residues 465 and 467 are phosphorylated [53;54]. Furthermore, the MH2-domain mediates the hetero-oligomerization with the other Smad proteins, the nuclear import and the binding of cofactors in the nucleus [28;32;50]. The MH1-domain of both R-Smads and Co-Smad is important for the nuclear localization, DNA-binding and also the binding of cofactors in the nucleus. The linker region can be phosphorylated by different kinases, and integrates input from other signaling pathways (see chapter 1.3.1.5). I-

Smads contain an MH2-domain, but the rest of the molecule is divergent from the other Smads, and they are not phosphorylated to become active. However, acetylation and ubiquitylation can modulate their activity [55;56]. The I-Smads have been implicated as transcription factors, regulating the expression of e.g. inhibitor of differentiation 1 (ID1) [32]. In addition, Smad7 has been demonstrated to bind the activated T β Rs as an adaptor protein and to facilitate non-Smad signaling [57].

1.3.1.3 *TGF- β -induced Smad signaling*

Upon binding of TGF- β to T β RII, which is a constitutively active serine-threonine kinase, T β RI is recruited and T β RII phosphorylates T β RI on a distinct glycine-serine-rich repeats (GS) domain [58]. In an unphosphorylated state the T β RI is known to interact with FKBP12, which blocks further signaling in the absence of ligand [59]. In addition, the T β RI remains in an inactive conformation, where the GS domain masks the kinase domain [60]. GS-domain phosphorylation reveals the masking of the kinase domain, thus makes further signaling possible [61]. FKBP12 stabilizes the inactive conformation of T β RI. Upon T β RI phosphorylation FKBP12 is released, and the T β RI can interact with and phosphorylate the intracellular signaling proteins. Crucial for the interaction between T β RI and R-Smads is the L45 loop in the kinase domain of the receptor [62] and the L3 loop in the MH2 domain of the R-Smads [63]. The affinity of the different Smad proteins in their interaction with the different type I receptors decides which Smads are activated. Generally, Smad2/3 are activated upon TGF- β stimulation [64;65], but Smad1 is shown to be activated via Alk-1 upon TGF- β stimulation in e.g. endothelial cells [66]. When the R-Smads are phosphorylated they dissociate from the receptors, cluster together in a heteromeric fashion [67], and form a complex with Smad4. This complex translocates to the nucleus, where it facilitates or inhibits gene transcription (Fig. 4) [28;32;50].

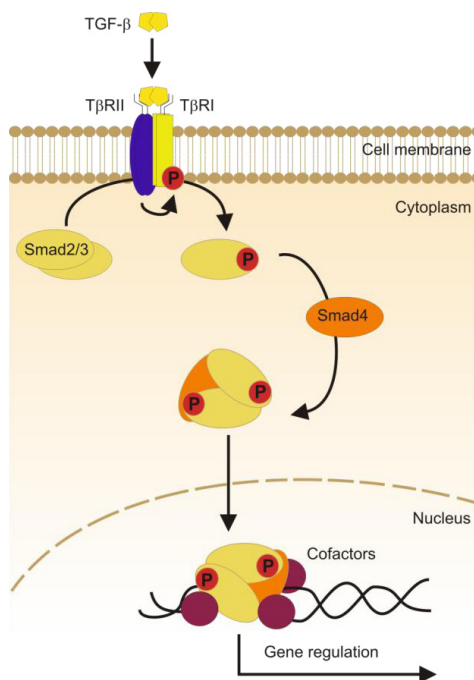


Figure 4. The canonical TGF-β signaling pathway

Dimeric TGF-β binds to a heterotetrameric receptor comprised of two type II receptors and two type I receptors. Upon ligand binding the type II receptors phosphorylate the type I receptors. This leads to activation of the intracellular Smad proteins, Smad2/3. Phosphorylated Smad2/3 can form a complex with Smad4. This complex translocates to the nucleus where it binds DNA together with different cofactors to regulate gene expression. (Modified from Schmierer and Hill, 2007 [28]).

1.3.1.4 Smads as transcription factors

The Smad complex binds to distinct DNA sequences situated at promoters or enhancers [28;32]. The DNA binding affinity of the complex is low, and Smad2 is not able to bind directly to the DNA due to an inserted sequence in the DNA-binding domain [68;69]. Smad3 has the highest affinity for the Smad-binding element (SBE), but can also bind to other sequences [28;32]. Several other transcription factors and co-activators in the nucleus are involved to initiate Smad-

dependent transcription. In addition, co-repressors can bind to the Smad complex, repressing transcription. The duration and strength of the signaling determines which genes are activated. The Smad proteins affect transcription of several target genes, by both up- and downregulation, to enable the many effects induced by the members of the TGF- β superfamily [32]. Important target genes are the Ids, which encode for inhibitory helix-loop-helix (HLH) proteins that can inhibit other basic HLH proteins [70]. The Id proteins lack a DNA-binding domain, but form heterodimers with other basic HLH proteins, thus inhibiting their function. Typical basic HLH proteins which are inhibited by the Ids are the E-proteins, e.g. E2A, and Ets and Retinoblastoma (Rb). Several of the Id target proteins are involved in the control of cell-cycle progression. Further TGF- β target genes include Pai-1 [71], c-Myc, p15 and p21, all of which, among other effects, affect cell proliferation [32]. In addition, inhibitors like Smad7 represent target genes that are upregulated in an autoinhibitory loop.

1.3.1.5 *Regulation of the TGF- β signaling pathway*

The TGF- β signaling pathway is thoroughly regulated to facilitate the fine-tuned mechanisms of TGF- β -mediated effects (Fig. 5). This is necessary for a potent morphogen. Post-translational modifications occur both at the receptor level [72] and in Smad proteins, of which phosphorylation and sumoylation primarily enhance and ubiquitylation primarily terminates the signaling [50]. Whether phosphorylation acts to enhance or inhibit signaling depends on which residues are phosphorylated. For example it has been suggested that phosphorylation of the linker region in R-Smads can inhibit further signal transduction [73]. In addition, these modifications are reversible, e.g. through protein phosphatases, which dephosphorylate phosphorylated residues to either terminate or facilitate signaling [74].

Several proteins have been found to bind to TGF- β or to the signaling proteins, e.g. the receptors (see chapter 1.3.1, 1.3.1.3 and 1.3.1.4 for examples). Of importance, the adaptor protein Smad anchor for receptor activation (SARA or ZFYVE9) is thought to play a role in bridging between the T β Rs and the R-Smads, thus facilitating R-Smad phosphorylation [75]. SARA, which harbors a FYVE domain, has been demonstrated to localize to early endosomes. The FYVE domain is a double zinc-finger motif that preferentially binds to phosphatidylinositol-3-phosphate

(PtdIns(3)P), which is enriched in endosomal membranes [76]. Disruption of this localization inhibits TGF- β -induced Smad2 nuclear translocation in HeLa cells [77]. It has therefore been suggested that internalization of the TGF- β receptor complex is important to induce signaling.

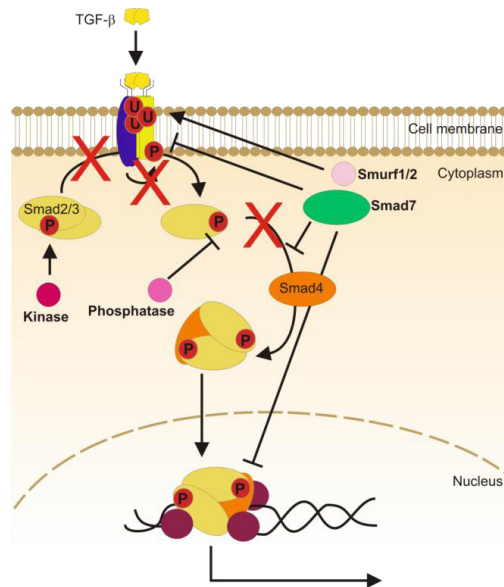


Figure 5. Regulation of the TGF- β signaling pathway

The TGF- β signaling pathway is tightly regulated. Smad7 is an inhibitory Smad that can inhibit the pathway at several levels. Smad7 also cooperates with Smurf1/2, which are ubiquitin ligases. This cooperation facilitates degradation of the TGF- β receptors. Furthermore, phosphatases can dephosphorylate the phosphorylated signaling components and this terminates the signaling. Kinases can phosphorylate residues in the linker region of Smad proteins, which hinders translocation of R-Smads to the nucleus. (Modified from Schmierer and Hill, 2007 [28]).

The I-Smads can act at several levels in the signaling pathway [78;79]. Smad6 is shown to preferentially inhibit BMP signaling [80], whereas Smad7 inhibits the signaling induced by

several of the TGF- β superfamily cytokines [81;82]. Smad7 can facilitate ubiquitylation of the T β Rs through the E3 ubiquitin ligases Smurf1 and Smurf2, compete with the R-Smads for binding to the type I receptors or inhibit the R-Smad-Smad4 complex formation [83]. Furthermore, Smad7 has been found in the nucleus preventing transcription of target genes by interfering with the DNA-binding of the Smad complex [50].

In addition to the intrinsic regulatory elements, other signaling pathways influence the TGF- β pathway on several levels [84]. This cross-talk is of high importance during embryogenesis, but also in tissue homeostasis in adults. Wnt, Notch, Hedgehog, PI3K/Akt and different mitogen-activated protein kinases (MAPKs), i.e. ERK1/2, p38 and Jnk, can phosphorylate proteins of the TGF- β signaling cascade. They have been found to phosphorylate the linker region of the R-Smads, which is rich in proline, serine and threonine residues. This can lead to attenuation but also to enhancement of signaling, depending on the kinase, which residue is modified and where in the signaling cascade the modification occurs. Thus, there can be different outcomes of e.g. Smad3 linker phosphorylation in the cytoplasm compared to in the nucleus [84]. Multiple phosphorylation can also lead to recruitment of Smurf1 and subsequent ubiquitination and degradation, as is known from Smad1 [85].

1.3.1.6 *Non-canonical TGF- β signaling*

In addition to the canonical TGF- β Smad signaling, TGF- β can also induce signaling through other molecules like p38 and ERK1/2 MAPKs (Fig. 6) [86;87]. Activation of ERK1/2 MAPK has been demonstrated to be important for epithelial-to-mesenchymal transition (EMT) [31], one of the tumor-promoting effects of TGF- β (see chapter 1.3.4.1). In addition, as discussed in chapter 1.3.1.5, ERK1/2 activation inhibits the canonical TGF- β signaling pathway [86;87]. Activation of p38 MAPK has been demonstrated to be important in the induction of apoptosis, together with the Smad proteins, and in the regulation of EMT. The activation of the MAPKs is found to be Smad independent. TGF- β -induced ERK phosphorylation occurs either rapidly, through direct activation of Ras, or more slowly, hours after TGF- β stimulation. This is proposed to be cell-type dependent. The direct activation of Ras is facilitated by tyrosine-phosphorylation of the T β Rs, recruitment of adaptor proteins like Grb2 and Shc, which subsequently activate Ras, a MAPK

kinase kinase, triggering the MAPK cascade [86;87]. p38 MAPK is activated via the MAPK kinase MKK3/6, which in turn is activated by TAK1, a MAPK kinase kinase that directly interacts with the T β RII together with tumor necrosis factor receptor-associated factor 6 (TRAF6) [86;87]. TRAF6, together with protein kinase C ζ (PKC ζ) and tumor necrosis factor- α converting enzyme (TACE), has recently also been implicated in cleavage of T β RI, leading to nuclear translocation of the cleaved intracellular domain [88]. This has been implicated to play a role in TGF- β -induced tumor promotion (see chapter 1.3.4.1).

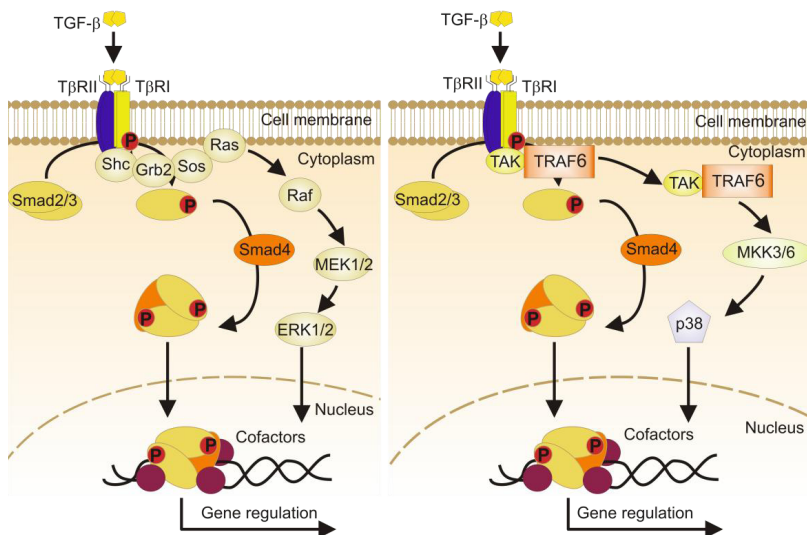


Figure 6. Non-canonical TGF- β signaling

This figure schematically depicts some of the non-canonical TGF- β signaling pathways. Upregulation of ERK1/2 and p38 MAPKs is independent of the Smad proteins. Adaptor proteins like Shc and Grb2 activate Ras, which in turn activates the MAPK signaling cascade, culminating in activation of ERK1/2 MAPK. p38 MAPK is activated via TAK1, TRAF6 and MKK3/6. (Modified from Schmierer and Hill, 2007 [28] and Zhang, 2009 [86]).

In addition to the Smad-independent signaling, the R-Smads, but not Smad4, have recently been implicated in microRNA (miRNA) processing in the nucleus [89]. Both TGF- β - and BMP-specific R-Smads have been shown to interact with the p68 protein, a subunit of the DROSHA RNase III, which is an important complex in the miRNA processing machinery.

1.3.2 Bone morphogenetic proteins

More than 20 members of the BMP subfamily exist in humans, making this the largest group within the TGF- β superfamily [90]. These proteins are highly conserved throughout species. All members have homologies, but some are more closely related. BMP-2 and -4 are cytokines with similar effects, which constitute one group, whereas BMP-6 and -7, together with BMP-5 and -8a/b, comprise another group within the family. The BMPs were discovered in 1965 by Urist, who demonstrated that new bone was able to grow from autoinduction of proliferating cells differentiating to osteocytes [91], hence the name BMP. BMPs are secreted as homodimers, with a few exceptions. As for TGF- β , some BMPs are non-covalently attached to its prodomain [90], and the attached prodomain can influence the bioactivity of the BMPs.

1.3.2.1 *BMP-mediated Smad signaling*

BMPs can bind to several of the superfamily receptors [90;92;93]. BMPs bind to BMP type II (BMPIIR), Activin type IIa (ActRIIa) and Activin type IIb (ActRIIb) of the type II receptors, and to Alk-1, Alk-2 (ActRI), Alk-3 (BMPRIa), Alk-4 (ActRIb) and Alk-6 (BMPRIb) of the type I receptors. The BMPs have different affinities for their receptors, and the type I and type II receptors can be combined in several ways. This contributes to the versatility of the BMP signaling. In contrast to TGF- β , most BMPs have higher affinity for the type I receptor, and the initial binding primarily occurs between a BMP and the type I receptor. However, binding to a preformed heterotetrameric receptor complex has been reported, e.g. for BMP-2 [94]. Binding of ligand to either a pre-formed receptor complex or a single receptor dimer is likely to induce different signaling pathways. In addition to the main receptors, several co-receptors are known, which either facilitate or inhibit signaling [90;92;93]. Decoy-receptors, e.g. BMP and activin membrane-bound protein (BAMBI), have extracellular domains resembling the type I receptors,

but they cannot signal as they lack the intracellular kinase domain, thus, they inhibit BMP activity.

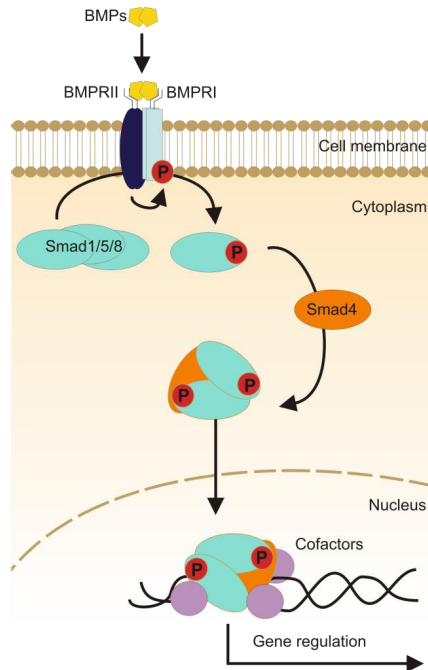


Figure 7. Canonical BMP signaling

This figure illustrates a simplified BMP signaling pathway. BMPs bind to a heterotetrameric receptor complex consisting of two type II and two type I receptors. The type II receptors phosphorylate the type I receptors, thus activating them. The type I receptors subsequently phosphorylate the intracellular R-Smads, Smad1/5/8, which can form a complex with Smad4. The complex translocates to the nucleus where it regulates gene transcription in concert with several cofactors. (Modified from Schmierer and Hill, 2007 [28]).

Upon ligand binding, the type II receptors phosphorylate the type I receptors [90;92;93] (Fig. 7). Among the R-Smads, Smad1/5/8 are important for BMP signaling [95-97]. However, as for TGF- β , certain BMPs have been demonstrated to induce phosphorylation of Smad2/3 [90]. The R-Smads form a complex with Smad4, and translocate to the nucleus. Smad1/5/8 preferably bind to guanine-cytosine-rich DNA sequences, but can also bind to the Smad-binding element (SBE) [92]. BMPs regulate many of the same target genes as TGF- β , and the ID genes are of high importance [98;99]. The negative feedback loop in this pathway includes upregulation of antagonists like Noggin, which is a target gene for many BMPs [93].

As for the T β Rs, the BMP receptors are internalized ligand-independently, but internalization with ligand via clathrin-coated pits and caveolae has also been demonstrated [90;93]. Whether signaling occurs from the cell surface, the endosomes, or both, is still unclear. It seems likely, that signaling is feasible both from the cell surface and from endosomes, however; more research is needed to elucidate the importance of both signaling modes. Internalization via clathrin-coated pits has been linked to the canonical Smad signaling, whereas internalization via caveolae has been linked to the non-canonical BMP signaling pathways. Whether this is strictly segregated is not known.

The BMP signaling pathway is under tight control, and many of the control mechanisms known from the TGF- β signaling pathway are involved (Fig. 8). Endofin (or ZFYVE16), another FYVE domain-containing protein, has been described as an adaptor protein facilitating BMP signaling [100]. BMP bioactivity is highly regulated by antagonists of which 15 are currently known, e.g. Noggin and Follistatin. The antagonists are divided into three subfamilies based on their cysteine-knot motif. They bind to BMPs, masking the epitopes which are responsible for receptor binding. Intracellularly, the I-Smads are important as a regulatory mechanism. Smad6 preferentially regulates BMP signaling, e.g. through interaction with type I receptors [80;101]. Smad7 also regulates the BMP pathway. In general, most regulatory elements are common for the BMP and TGF- β signaling pathways (see chapter 1.3.1.5).

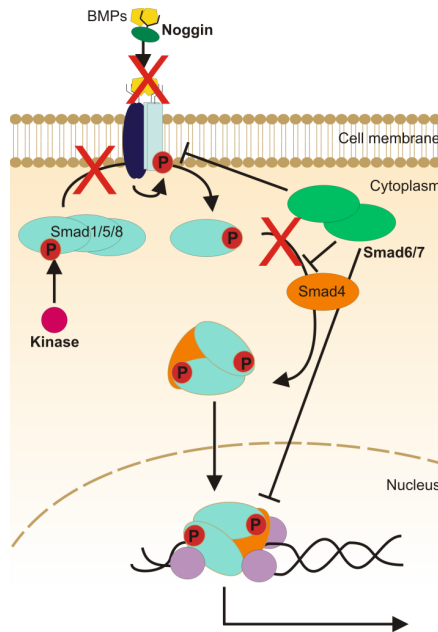


Figure 8. Regulation of the BMP signaling pathway

The BMP signaling pathway is highly regulated. Extracellular regulation of the BMPs' bioavailability is enabled by antagonists such as Noggin. Intracellular regulation is controlled by Smad6/7, which inhibit the pathway at several levels. In addition, kinases can phosphorylate the Smad proteins on residues in their linker regions; which hinders translocation of the R-Smads to the nucleus. (Modified from Schmierer and Hill, 2007 [28]).

BMPs can also induce non-canonical signaling, resulting in activation of many of the same proteins as for TGF- β [90;93]. However, some of the adaptor proteins differ. X-linked inhibitor of apoptosis protein (XIAP) and BMP receptor-associated molecule 1 (BRAM1) have been reported to bind BMP receptors and induce e.g. p38 and Jnk MAPKs activation.

1.3.3 Activins and their signaling

Activins are also secreted as dimers, constituted of two β -subunits [102]. In humans four different β -subunits have been identified, of which all can be combined as dimers. A range of different types of active activin dimers have been described. They can bind two distinct type II receptors, ActRIIa or ActRIIb, and two distinct type I receptors, Alk-4 or Alk-7, of which Alk-4 is the most common receptor. Activation of these receptors generates signaling through Smad2/3 and Smad4. Like other TGF- β superfamily cytokines, non-canonical signaling through activation of p38 and Jnk MAPKs has been demonstrated. As activins are also major regulators of cell proliferation and apoptosis, many of their target genes are identical to those of TGF- β and BMPs. Regulation of the bioavailability is mediated by antagonists like Follistatin in the extracellular space. Regulation of the signaling pathway involves many of the same mechanisms as for TGF- β and BMPs, e.g. Smad7 and modifications exerted by proteins from other pathways.

1.3.4 The TGF- β superfamily consists of morphogens with pleiotropic effects

All members of the TGF- β superfamily are highly conserved throughout species, and exert their many effects on a vast range of cell types, including development of hematopoietic cells [103] and immune cells in adults [104]. The effects of TGF- β on immunity depend on the differential state of the cells affected and on the cytokine milieu affecting the same cells. Generally, TGF- β inhibits T-cell differentiation, induces regulatory T (T_{reg}) cells and hampers antigen presentation as a negative regulator of inflammatory immune responses. However, recruitment of leukocytes in the initial phase of an immune response and pro-survival effects on differentiated T cells are also known outcomes. Less is known about the effects of BMPs on T cells. BMPs have been demonstrated to inhibit early thymocyte differentiation [105] and BMP-6 induced growth-inhibitory effects on mature T cells [106].

TGF- β -induced effects on B lymphocytes include growth inhibition through cell-cycle arrest and induction of apoptosis in addition to a general inhibitory function on antibody production [104;107]. However, TGF- β is also known to induce IgA class switching in B cells. In more detail, TGF- β is known to inhibit cell growth through inhibition of G1- to S-phase transition [108], and to induce apoptosis by regulating members of the intrinsic apoptotic pathway [109].

Induction of apoptosis has also been reported to occur via the lipid phosphatase Src homology 2 domain-containing 5' inositol phosphatase (SHIP) [110]. Regarding the BMPs, BMP-6 has been shown to inhibit the growth of mature human B cells [111] and to inhibit human bone marrow B lymphopoiesis [112]. Moreover, Huse and colleagues demonstrated that several BMPs inhibit the Ig-production of B cells [113]. From the general inhibitory effects of TGF- β on the immune system follows an inhibition of the host tumor immune surveillance, which is favorable for tumor progression [114]. This is in addition to the development of resistance to TGF- β and BMPs in the malignant cells.

1.3.4.1 *TGF- β and BMPs – tumor suppression and tumor promotion*

As TGF- β and BMPs exert effects to inhibit cell growth, they are important cytokines to fight the development and growth of cancers [115;116]. However, many cancer types have developed ways to escape the growth inhibition. The escape mechanisms range from bi-allelic inactivation of central components of the signaling pathways, e.g. T β RII, to upregulation of inhibitors, like Smad7. Mutations in the T β RII gene, which lead to either a truncated or a kinase-dead protein, have been demonstrated in several cancer types, e.g. colon and ovarian cancer [115;116]. These mutations are frequently found in cancers with microsatellite instability. Microsatellite instability is a high mutation rate in certain short repetitive DNA sequences spread throughout the genome, called microsatellites [117]. Mutations in the type I receptors Alk-3, Alk-4 and Alk-5 are found in colon, pancreatic and breast cancer, respectively, but alterations in the type II receptors are more common [115;116]. Smad4 mutations or deletion of one Smad4 allele are highly present in many cancer forms, such as pancreatic and colorectal cancer types. R-Smad mutations are less frequent, but deletion of Smad3 has been demonstrated in gastric cancer [115;116]. Overexpression of Smad7, as seen in the colonic mucosa, can be associated with chronic inflammation, rendering the tissue predisposed to develop cancer.

At a certain point in the development of cancer, TGF- β can promote cancer growth [118;119]. The switch in responsiveness leads to lack of growth inhibition and other TGF- β effects become more pronounced, e.g. EMT, motility, invasion and immunosuppression [118;119]. Many of these effects can lead to the development of metastasis, e.g. EMT, which is the process where

epithelial cells lose their anchorage to other cells and gain the ability to move to a different location. The mechanisms behind the switch in TGF- β responsiveness involve both genetic and epigenetic changes. Mutant p53 and oncogenic Ras have been demonstrated to enable TGF- β -induced metastasis via repression of p63 in breast cancer cells [120]. Another mechanism involves loss of Smad4 in colon cancer cells, which facilitates liver metastases [121]. Restoring Smad4 expression promotes the TGF- β tumor-suppressive effects in these cells. In addition, the process of EMT leads to cells with “stem-cell properties” [122]. This is a possible answer to the question of how metastasizing cells are able to colonize new tissue. Non-stem cells have only limited proliferative potential; however, when the EMT program induces “stem cell-properties”, the growth of metastases is explainable. Overexpression of TGF- β has been found in different cancers, e.g. breast and colon, and is linked to metastasis [115]. TGF- β can be secreted by tumor cells, stromal cells or tumor-infiltrating cells, thus there are many sources of TGF- β in a tumor setting. BMPs have also been implicated to have dual roles in cancer; however, additional research is needed to address this issue more thoroughly [123;124]. High expression of BMP-6 in myeloma patients has been linked to superior survival compared to the low BMP-6-expression group [125]. On the contrary, another study demonstrated that BMP-6 protected against apoptosis in myeloma cell lines, thus promoting survival of myeloma cells [126]. This indicates that BMPs can have tumor-suppressing and tumor-promoting effects in hematologic malignancies.

1.3.4.2 *Roles in hematologic malignancies – resistance to BMPs and TGF- β*

In leukemia or lymphoma cell lines, resistance to the antiproliferative effects of TGF- β and BMPs is frequently seen. Mutations in central components, e.g. receptors or Smad proteins, are rare, although reported in some cases of T-cell lymphomas and anaplastic large-cell lymphomas [127;128]. Reduced expression of central signaling components or overexpression of oncogenes is more common. In BL, resistance to TGF- β has been linked to a reduction in the T β RII cell-surface expression [129;130]. This was most abundant in BL cell lines expressing all the EBV genes, and it was not due to a mutation [129]. These cell lines were found refractory both to the antiproliferative and apoptotic effects of TGF- β . Importantly, EBV has previously been associated with loss of TGF- β responsiveness [131]. Reduced Smad3 protein level has been demonstrated in patients with T-cell lymphoblastic leukemia [132]. Moreover, loss of one Smad3

allele in mice was linked to impaired TGF- β antiproliferative effects. Loss of Smad3 together with inactivation of the p27 gene was demonstrated to promote leukemogenesis in mice. Promoter methylation of the BMP-6 gene has been found in malignant lymphoma and adult T-cell leukemia [133;134]. This may be a predisposition for developing malignant disease, and can be associated with aggressiveness of the malignancy. In addition, promoter methylation of T β RII was suggested in a DLBCL cell line, as treatment with demethylating reagents restored the expression and functionality of the receptor [135]. This demonstrates that epigenetic silencing through DNA methylation is a factor in the resistance to members of the TGF- β superfamily in hematologic malignancies.

1.4 Epidermal growth factors

Epidermal growth factor (EGF) is a mitogenic factor that plays an important role in the regulation of cell growth, proliferation and differentiation [136;137]. EGF was first discovered in mice and later the equivalent was detected in humans. The EGF family of growth factors consists of seven different growth factors, e.g. EGF and TGF- α . There is limited homology within the family; however, they all have an EGF-like domain and bind the same family of receptors.

1.4.1 EGF receptor activation and internalization

EGF binds to the epidermal growth factor receptor (EGFR, also known as ErbB1), a tyrosine-kinase receptor belonging to the ErbB family of receptors [138]. Upon ligand binding the receptors form either hetero- or homodimers and are autophosphorylated on tyrosine residues. Following activation, the receptor complex is internalized via clathrin-coated pits to early endosomes, where its fate is decided [138]. Either it is recycled back to the cell surface, or, more commonly, ubiquitinated and sorted into multivesicular bodies and targeted for degradation in the lysosomes. Signaling can occur both from the cell surface and from the endosomes [139;140].

2. Aims of the present study

The TGF- β and BMP signaling pathways seem, at first glance, straightforward. However, research over the past years has revealed several control mechanisms and also non-canonical signaling, clarifying some of the complexity of these pathways. In cancer, alterations in the signaling pathways occur to give growth advantage to the cancerous cells and understanding the mechanisms behind these alterations are of high importance.

The overall aim of our studies was to elucidate the TGF- β and BMP signaling pathways in B-cell lymphoma, focusing on the mechanisms behind development of resistance and revealing the role of key players in these pathways. In more detail, the aims of the studies were to:

- Investigate whether B-cell lymphomas can escape the growth control of BMPs, and, if found, elucidate the molecular mechanisms behind the resistance.
- Study the mechanisms behind resistance and sensitivity to TGF- β -induced growth inhibition in B-cell lymphomas.
- Elucidate the role of Smad anchor for receptor activation (SARA) in the TGF- β signaling pathway.

3. Summary of included papers

Paper I: Mechanistic basis for resistance to BMP-induced growth inhibition in B-cell lymphoma

BMP expression and signaling are altered in a variety of cancers but the functional impact of these alterations is uncertain. In this study we investigated the impact of expression of BMP-2, -4, -6 and -7 and their signaling pathway components in human B-cell lymphoma. BMP mRNA levels were determined with real-time RT-PCR. High levels of BMP-7 mRNA were detected in both normal GC B cells and malignant B cells. In addition, some BMP-6 mRNA was detected. Furthermore, we tested the functional effects of the BMPs on B-cell lymphoma cells. Addition of exogenous BMPs inhibited DNA synthesis in most lymphoma cell lines examined but some cell lines were completely resistant. Notably, BMP-7 had little if any effect in all lymphoma cells tested. Primary tumor specimens from three out of five lymphoma patients examined were resistant to BMPs, as determined by a lack of activation of the BMP effectors Smad1/5/8. BMP-resistance mechanisms were investigated by a comparison of sensitive and resistant cell lines. In this respect we investigated the levels of Smad1/5/8 phosphorylation in the sensitive and resistant cell lines through Western immunoblotting. We found a positive correlation between activation of Smad1/5/8 and inhibition of DNA synthesis. This suggests that the mechanism behind resistance is situated upstream of Smad activation. While BMP receptors are downregulated in many cancers, we documented similar receptor levels in both resistant and sensitive lymphoma cells. We investigated whether Smad levels could be a general resistance mechanism; however, only one of the resistant cell lines displayed downregulated Smad1/5 levels. Upregulation of I-Smad proteins has been demonstrated as a mechanism behind resistance in different cancer types. In primary lymphoma specimens from patients, analysis of two independent gene expression data sets confirmed elevated expression of Smad7. In the resistant cell line ROS-50 we observed elevated levels of Smad7 mRNA; however, we did not detect the same difference on the protein level. Overexpression of Smad7 in the highly sensitive cell line SUDHL-6 rendered the cells resistant to the growth-inhibitory effects of BMPs, thus, Smad7 upregulation is a potential mechanism behind resistance to BMPs in B-cell lymphoma. Our findings define a mechanism of escape from inhibitory BMP signaling in B-cell lymphomas expressing BMPs that is correlated to reduced activation of Smad1/5/8.

Paper II: TGF- β -induced growth inhibition in B-cell lymphoma correlates with Smad1/5 signalling and constitutively active p38 MAPK

In this paper we compared the TGF- β -related signaling properties in B-cell lymphoma cell lines that were sensitive or resistant to TGF- β -induced antiproliferative effects. TGF- β -sensitive cell lines expressed higher cell-surface levels of Alk-5, determined by flow cytometry. The expression levels of the other TGF- β and BMP receptors were comparable in the different cell lines. Furthermore, we investigated the levels of Smad2 and Smad1/5 phosphorylation upon TGF- β treatment by Western immunoblotting. TGF- β -induced phosphorylation of Smad2 was similar in TGF- β -sensitive and -resistant cell lines. In contrast, activation of Smad1/5 was restricted to cells that were sensitive to growth inhibition by TGF- β . Moreover, with activin A we detected limited antiproliferative effects, strong phosphorylation of Smad2, but no Smad1/5 phosphorylation. Upregulation of the TGF- β target genes Id-1 and Pai-1 was identified in the TGF- β -sensitive cell lines. The importance of the non-canonical TGF- β signaling pathways has recently emerged, thus, we wanted to examine whether these pathways could play a role in the sensitivity and resistance to TGF- β . Constitutive phosphorylation of p38 MAPK was restricted to the TGF- β -sensitive cell lines. Inhibition of p38 MAPK led to reduced sensitivity to TGF- β . In addition, we demonstrated constitutive active ERK1/2 MAPK expression in the TGF- β -resistant cell lines. We suggest that phosphorylation of Smad1/5 is important for the antiproliferative effects of TGF- β in B-cell lymphoma. Alk-5 was highly expressed in the sensitive cell lines, and might be important for signaling through Smad1/5. Our results further indicate a role for p38 MAPK in the regulation of TGF- β -induced antiproliferative effects.

Paper III: SARA has a role in EGF receptor trafficking, but is not essential in TGF- β signaling

Signaling through receptors of the TGF- β superfamily is mediated by Smad proteins. It has been suggested that the FYVE-finger domain-containing protein SARA influences TGF- β signaling by direct interaction with the non-activated Smad proteins and the TGF- β receptors, thus forming a bridge between the receptors and R-Smads to facilitate signaling. The specific role of SARA in TGF- β -mediated Smad signaling remains poorly understood. We studied SARA protein expression in different B-cell lymphoma cell lines by Western immunoblotting, but did not find any correlation between SARA expression levels and the levels of TGF- β -induced phosphorylation of Smad proteins. Knockdown of SARA in HeLa cells by two different siRNAs did not interfere with the TGF- β -induced Smad activation or Smad nuclear translocation. Furthermore, we performed real-time RT-PCR to investigate the levels of target-gene induction, and detected no difference in the induction of TGF- β target genes ID1, ID2 and ID3 between control and SARA knockdown cells. As determined by a [3 H]-thymidine-incorporation assay and live-cell microscopy, ablation of SARA resulted in reduced cell proliferation and cell count, which led us to investigate a potential role of SARA in regulation of EGFR trafficking. We did confocal microscopy looking at the uptake and sorting of rhodamine (Rh)-EGF. Knockdown of SARA showed significant impact on the sorting of Rh-EGF. Furthermore, EGF-induced internalization and degradation of the EGFR was reduced in SARA knockdown cells compared to control cells, as determined by flow cytometry and Western immunoblotting, respectively. Our results fail to support that SARA is essential for TGF- β -mediated signaling, but instead suggest a novel role for SARA in the regulation of EGFR trafficking.

4. Discussion

4.1 Methodological considerations

4.1.1 Cell systems

We have used primary tumor material, centroblasts and centrocytes isolated from human tonsils, B cells isolated from peripheral blood from healthy donors and B-cell lymphoma cell lines. These cell systems have advantages and disadvantages with respect to being good models for B-cell lymphomas and their corresponding normal controls and, at the same time, rendering it possible to use them for laboratory work. Primary tumor material is undoubtedly the best model for B-cell lymphoma; however, tumor material is not easily accessible, the cells are fragile and the amount of cells from each biopsy is scarce, which limits the experiments that can be conducted. In primary tumor material non-tumor cells are always present, and purification of tumor cells reduces the viability of the cells and leads to a possible further selection of the cells. In addition, primary cells need to be stimulated with survival factors, e.g. CD40L and different cytokines, to survive. Moreover, there are ethical and juridical considerations when working with patient material. In this respect, the work done on patient material, cells isolated from human tonsils and B cells derived from healthy donors was approved by the regional Committee for Medical Research Ethics, Region Eastern Norway. Informed consent from each donor was obtained, in accordance with the Declaration of Helsinki [141]. Primary tumor cells can be used to confirm results obtained from cell lines to ensure the physiological importance of the findings, which we did in paper I.

In paper I we used centroblasts and centrocytes as controls, and in paper II we used peripheral blood bulk B cells. Centroblasts and centrocytes from tonsils are considered to be the normal counterparts for certain lymphoma subtypes, e.g. BL, FL and GCB DLBCL. However, these cells are more difficult to get access to, compared to e.g. peripheral blood B cells, and as tonsils are removed only when patients are suffering from inflammation, the cells might not be considered “normal”. Interestingly, Alizadeh and colleagues defined that the ABC DLBCL corresponds to

the activated peripheral B cells [24], rendering peripheral blood bulk B cells suited as a normal counterpart.

Several of the cell lines that we have used have been thoroughly tested and are considered good models for B-cell lymphoma subtypes, including SUDHL-4, SUDHL-6, Oci-Ly 3, Oci-Ly 7 and Oci-Ly 10 [24;142-144]. Others, like the FL cell lines, might not be as representative for the primary tumor. *In vivo*, FL cells are dependent on stromal cells to grow and the microenvironment has been demonstrated to be of high importance in the FL pathogenesis [25;145]. *In vitro* they grow without stromal cells; which means the cells must have obtained novel features compared to their cells of origin. Nevertheless, cell lines are easy to culture, the amount of cells is almost unlimited, cell lines are generally stable, which means the results are reproducible, and the cells are often easy to manipulate genetically. Thus, cell lines are good tools for researchers. However, their physiological relevance is debatable, and results often need to be confirmed in primary cells. Generally, there is a need for carefulness when working with cell lines, especially considering cross-contaminations and misidentification [146]. During this study, we have checked all the applied cell lines and have not experienced problems with e.g. mycoplasma contamination. As a rule, we only maintained our cell lines in culture for two months before new cells were thawed, to avoid genotypic and phenotypic changes. The cell lines SUDHL-4, SUDHL-6, ROS-50, K-422, BL-41 and Ramos were authenticated in February 2011 through DNA profiling by RT-PCR of 16 polymorphic markers. Regarding Oci-Ly 3, Oci-Ly 7 and Oci-Ly 10, these cell lines are not commercially available; thus, there is no cell-line identification profile available. However, we obtained these cell lines from Louis Staudt's lab, and they have profiled and classified them.

4.1.2 Manipulation of B cells and B-cell lymphoma cell lines

Transfection of normal B cells and several of the B-cell lymphoma cell lines used in this study has proven difficult. When attempting to transfect normal B cells using the nucleofection technique by Amaxa, we experienced low transfection efficacies and high proportions of cell death, despite the fact that others have succeeded in transfecting primary human B cells and B-cell chronic lymphocytic leukemia cells [147]. In addition, we experienced the same difficulties

with several of the B-cell lymphoma cell lines. This left us with few choices considering manipulation of the cells. Inhibitors can to some degree replace the use of siRNAs, but the inhibition achieved is not as good over time. In addition, small-molecule inhibitors are generally less specific compared to siRNA. The degree of off-target effects often correlates with the concentration of the applied inhibitor. Nevertheless, we successfully used the p38-inhibitor SB203580 in paper II, showing that p38 MAPK is important for the effects of TGF- β on cell death. We have recently established retroviral transduction in our lab, and this will open up more possibilities when it comes to manipulation of B cells and B-cell lymphoma cell lines.

In paper III we decided to apply the epithelial cancer cell line HeLa when elucidating the role of SARA in the TGF- β signaling pathway. These cells are easy to transfect using lipofectamin. In addition, they express higher levels of SARA compared to B-cell lymphoma cell lines, and they respond to TGF- β via both Smad2 and Smad1/5 phosphorylation. HeLa cells have a favorable morphology with a large cytoplasm, which is ideal for confocal microscopy, one of the techniques used in paper III.

4.1.3 Confocal microscopy

Antibodies applied to confocal microscopy need to be tested, as unspecific staining easily can occur. The rabbit anti-Smad2 antibody which we applied for confocal microscopy in paper III has been used for Western immunoblotting analysis as well, and is a specific antibody. We used rabbit antiserum as a negative control for the Smad2 staining.

4.1.4 Western immunoblotting quantification

The use of Western immunoblotting analysis relies on having antibodies with high specificity. The advantage of the method is that the proteins are separated by electrofocusing, thereby giving size information. Quantification of the protein bands is a method to objectively quantify the expression levels. However, it is important to be precautionous when quantifying blots. It is crucial to use a good loading control that the protein band of interest can be normalized to. We have used PGK-1, Actin, β -tubulin or the unphosphorylated corresponding protein as loading controls for

our Western blots. PGK-1, Actin and β -tubulin are housekeeping genes, and these are normally expressed at a constant level [148]. Greer and colleagues concluded that Actin, together with heat-shock protein 90 (Hsp-90), are among the more stably expressed housekeeping genes. It is also essential not to expose the Western blot film more than necessary, as the bands quickly may reach a point of saturation. Beyond this point the peaks of the bands are cut off, which leaves the differences smaller when compared to unsaturated bands. We have used a GS-300 densitometer (BioRad) and the software Quantity One when quantifying the protein bands. This software can be programmed to detect saturated bands upon scanning the films. Note that none of the protein bands we have quantified were saturated. In addition, it is important to repeat the experiments several times to be able to draw a reliable conclusion.

Many of the proteins in the BMP and TGF- β signaling pathways, including the cytokines themselves and the I-Smads, Smad6/7, are highly conserved throughout species. This reduces the immunogenicity of the proteins; therefore, the production of specific antibodies is challenging. We have experienced that several of the BMP-6, BMP-7 and Smad7 antibodies commercially available were not specific. We have tested the specificity of several of the antibodies used via pre-incubation with a blocking peptide or overexpression of a tagged protein. We used the latter experimental design to validate anti-human Smad7 and BMP-7 antibodies.

4.2 Elucidating the TGF- β and BMP signaling pathways in hematologic malignancies – sensitivity or resistance to the growth-inhibitory effects

Understanding the TGF- β and BMP signaling pathways in malignant cells is of high importance, and might identify aberrant expression levels of proteins that can represent new targets for therapeutic interventions in malignancies, including B-cell lymphomas. TGF- β and BMPs induce pleiotropic effects on a plethora of cell types, and the functional outcome is highly cell-type and context dependent. Research over the past years has revealed layers of control mechanisms that regulate the many effects. In paper I-III we have aimed to elucidate the BMP and TGF- β signaling pathways and how malignant B cells can develop resistance to these cytokines. Malignant cells often develop resistance to growth-inhibitory cytokines like TGF- β and BMPs. Both in paper I and II we demonstrated resistance to the growth-inhibitory effects of BMPs and

TGF- β , respectively. Three out of ten B-cell lymphoma cell lines were resistant to BMPs growth inhibition, whereas three were classified as sensitive and four displayed intermediate sensitivity. Five out of eight B-cell lymphoma cell lines were sensitive to TGF- β growth inhibition, albeit not as sensitive as normal B cells, whereas three were completely resistant. Upon further investigations we concluded that BMP and TGF- β resistances could be mediated by different aberrations in the signaling proteins.

4.2.1 Receptor downregulation is not a general mechanism behind the BMP and TGF- β resistances in B-cell lymphoma

In paper I and II we detected moderate levels of most BMP and TGF- β receptors; however, at least one type I and one type II receptor were detected in all cell lines. The receptor expression differed throughout the cell lines; however, we did not detect a pattern in sensitive compared to resistant cell lines, indicating that receptor downregulation is not a general mechanism behind the resistance to BMPs and TGF- β . Loss of TGF- β receptor expression has been reported in some cases of BL and ABC DLBCL [129;130;135]. We detected very low BMP receptor levels in some cell lines (paper I), especially of the type II receptors. Raji, a BMP-sensitive cell line, expressed low levels of type II receptors; however, it must be sufficient for signaling, as we detected Smad phosphorylation and growth-inhibiting effects after addition of BMPs to this cell line. Of note, we used flow cytometry to examine the levels of endogenous receptors. This method has detection limits, so most likely there are receptors expressed on the cells that were not detected in these experiments. However, we previously obtained similar results when comparing protein detection with flow cytometry to Western immunoblotting [106], thus verifying that flow cytometry can be used to detect BMP and TGF- β receptors. In paper II we show that Alk-5 and T β RII are expressed in the cell lines, and, most likely, features signaling via both Smad2 and Smad1/5. The expression of Alk-5 correlated with sensitivity to TGF- β as the sensitive cell lines expressed higher surface levels of Alk-5 compared to the resistant cell lines. Regarding the BMPs, research over the past decades has demonstrated that each BMP has binding preferences to certain receptor types, which they bind to with higher affinity [90]. Therefore, each cell's composition of the type I and II receptors will affect the functional outcome of different BMPs in that cell. BMP-2 has been shown to induce proliferation of different cell types, including prostate cancer cells, possibly due to the type I receptor present on

the cells, as upregulation of BMPRII led to antiproliferative BMP-2-induced effects. [149]. It is probably dependent on the cell type and the levels of both receptors and ligand. The BMPs that have been used in this study all bind to Alk-2, Alk-3 and Alk-6, and to BMPRII, ActRIIa and ActRIIb; however, with different affinities [90;92].

We detected BMP- and TGF- β -receptor expression in our lymphoma cell lines, and concluded that downregulation of receptors is not the reason for development of resistance. However, we did not elucidate whether the expressed receptors harbored mutations. This is likely in cell lines expressing receptors without being able to induce phosphorylation of R-Smads. Taken together, downregulation of receptors is not a general mechanism behind the resistance to BMPs and TGF- β in B-cell lymphoma.

4.2.2 Smad1/5/8 signaling is linked to the antiproliferative effects of BMPs and TGF- β

Different adaptor proteins, e.g. SARA, have been proposed to have important roles in facilitating Smad phosphorylation by the T β RI, and loss of SARA expression could be a potential mechanism of resistance to the growth-inhibitory effects of TGF- β . We demonstrated that SARA expression does not correlate to TGF- β -induced antiproliferative effects or Smad phosphorylation in B-cell lymphoma cell lines (paper III). Therefore, loss of SARA is not a mechanism behind resistance to TGF- β . In addition, knockdown of SARA did not affect TGF- β signaling in HeLa cells. Whether loss of Endofin or other adaptor proteins might be linked to resistance to BMPs or TGF- β in B-cell lymphoma has to our knowledge never been elucidated and needs to be further investigated. The role of SARA and other adaptor proteins will be further discussed in chapters 4.4 and 4.5.

The canonical TGF- β -induced signaling is generated through Smad2/3; however, recent research has demonstrated that TGF- β also can activate Smad1 or 5 in several cell types [150;151]. In paper II and III we revealed that TGF- β could induce phosphorylation of Smad1/5 in normal and neoplastic B cells. The TGF- β -resistant cell lines signaled through Smad2 only, whereas the

sensitive cell lines signaled through both Smad2 and Smad1/5. In addition, in paper I we demonstrated a correlation between Smad1/5/8 signaling and the antiproliferative effects of BMPs on B-cell lymphoma cell lines. Thus, the Smad1/5/8 signaling is of importance to the growth-inhibitory effects of both BMPs and TGF- β . Munoz and colleagues also discovered that Smad1 is important for TGF- β signaling in FL and DLBCL cell lines [152]. They observed that TGF- β signaling involving T β RII can phosphorylate Smad1, and that this phosphorylation is important for cell proliferation of one FL cell line as the antiproliferative effects of TGF- β were diminished after transfection of siRNA against Smad1. In addition, Rai and colleagues demonstrated that Smad5 is inhibited via miRNA miR-155, and this affects the TGF- β antiproliferative effects [153], showing that signaling through Smad5 is important for the functionality of TGF- β . Another study confirmed TGF- β Smad1/5 signaling in mammary epithelial cell lines; however, they detected a correlation to the migratory effects of TGF- β , not to the antiproliferative effects [154].

In paper I and II we demonstrated that the applied B-cell lymphoma cell lines expressed both R-Smads and Smad4 at levels sufficient to signal. Although the R-Smad levels differed, we did not detect expression patterns distinguishing between resistant and sensitive cell lines. In K-422 cells the combined expression of Smad1/5 was lower than in the other cell lines (paper I). We succeeded to overexpress Smad1 via electroporation of K-422 cells, but Smad1/5 phosphorylation was not restored, and the cells did not become sensitive to BMPs (unpublished data). However, electroporation might alter the signaling capacity of the cells, which makes it more difficult to interpret the results. In conclusion, altered Smad levels is most likely not the mechanism behind resistance to BMPs or TGF- β in K-422 cells; however, cannot be excluded as a mechanism as we observed downregulation of Smad1 in some B-cell lymphomas (paper I).

Resistance to the growth-inhibitory effects of BMPs and TGF- β has been linked to the Smad proteins. Mutations in Smad proteins are rarely found in B-cell lymphoma; however, suppression of Smad signaling has been demonstrated both via miRNA targeting Smad5 and upregulation of BCL6 [153;155]. BCL6 is frequently upregulated in FL and DLBCL, and Wang and colleagues

detected that upregulation of BCL6 correlated with resistance to TGF- β -induced growth inhibition [155]. BCL6 was found to interact with Smad4, thus repressing TGF- β signaling. We tried to elucidate the role of BCL6 in TGF- β signaling in B-cell lymphoma. From our preliminary studies we came to the conclusion that BCL6 most likely is not involved in abrogation of TGF- β signaling in our cell lines (unpublished data). In recent years, the role of miRNAs has become more and more clear, and changes in miRNA expression are often linked to malignancies. Rai and colleagues demonstrated that miR-155 targeted Smad5, and thus limited the cytostatic activity of both BMPs and TGF- β [153]. We did not elucidate the role of miRNAs in B-cell lymphoma.

In myeloma cells, a distinct mechanism for TGF- β resistance was demonstrated. Cyclin-dependent kinase 2 (CDK2) was found to phosphorylate Smad2 on a threonine residue (T8) in the amino-terminal end, leading to inhibition of Smad2-Smad4 complex formation and abrogated nuclear translocation [156]. Baughn and colleagues stated that they detected Smad2 phosphorylated on the T8 residue in the two lymphoma cell lines Ramos and BJAB [156]. However, in paper II we show that Ramos cells are sensitive to TGF- β antiproliferative effects, albeit not to the same degree as normal B cells. It is not clear whether Baughn and colleagues tested the functional effects of TGF- β on lymphoma cell lines, or just examined the T8 phosphorylation status of Smad2. Nevertheless, phosphorylation of T8 might contribute to the reduced sensitivity to TGF- β that we see in Ramos cells. Studies of other cell types have demonstrated that phosphorylation of Smad3 by both CDK2 and CDK4 also attenuates TGF- β signaling, and leads to resistance to the antiproliferative effects of TGF- β [157]. We have not explored the role of R-Smad phosphorylation by CDKs in our cell lines. Taken together, downregulation of Smad proteins is not a general mechanism behind loss of the antiproliferative effects of BMPs and TGF- β in B-cell lymphoma; however, might play a role in certain B-cell lymphomas. In addition, signaling through Smad1/5/8 is crucial for the growth-inhibitory effects of BMPs as well as TGF- β in B-cell lymphoma. The mechanisms behind the resistance to BMPs are most likely situated early in the signaling cascade, as we detected a correlation between Smad1/5/8 phosphorylation and sensitivity to BMP-induced growth inhibition (paper I).

4.2.3 Inhibitory Smad proteins and antagonists are potential mechanisms behind BMP and TGF- β resistances

As seen in solid cancers, upregulation of Smad7 has been found in adult T-cell leukemia/lymphoma [158]. Nakahata and colleagues demonstrated that overexpression of Smad7 together with downregulation of the transcription factor ZEB1 were the mechanisms behind resistance to TGF- β in adult T-cell leukemia/lymphoma. In paper I differences in the mRNA expression level for Smad7 were detected; however, we could not detect protein expression patterns distinguishing between resistant and sensitive cell lines. The correlation between the mRNA and protein data was poor. This is probably due to post-transcriptional regulation, as is known from e.g. TGF- β transcription [159]. SUDHL-6 cells showed slightly lower Smad7 protein levels compared to the other cell lines. In addition, these cells were by far the most BMP sensitive; therefore, we used these cells to stably overexpress Smad7. Smad7 overexpression rendered the cells BMP resistant (paper I), thus, Smad7 upregulation is a potential mechanism behind both BMP and TGF- β resistances in B-cell lymphoma.

Another mechanism implicated in the escape from BMP-mediated growth inhibition is upregulation of the soluble antagonist Noggin. In association with Paper I we determined the mRNA levels of Noggin and other BMP-inhibitors in B-cell lymphoma cell lines. We did not detect increased levels of any of the tested inhibitors; rendering it unlikely that upregulation of inhibitors is a mechanism behind escape from BMP-induced growth inhibition in lymphoma cells (unpublished data). However, upregulation of Noggin has been demonstrated as a mechanism behind resistance to BMPs in different cancer types. Hsu and colleagues detected upregulation of Noggin in aggressive melanoma cells that were refractory to the BMP-7-induced antiproliferative effects [160]. In some melanoma cell lines, overexpression of Noggin led to reduced BMP-7-induced growth inhibition, which correlated with induced expression of two growth factors, Nodal and Vascular endothelial growth factor (VEGF). This can in part be responsible for the reduced antiproliferative effects. Taken together, the upregulation of inhibitors is a potential mechanism behind the resistance to the BMP- and TGF- β -induced growth inhibition. However, in B-cell lymphoma we did not detect high levels of antagonists like Noggin. Moreover, we demonstrated that Smad7 overexpression can render a cell line BMP resistant, thus, Smad7 upregulation is a potential mechanism behind BMP and TGF- β resistances.

4.2.4 Non-Smad signaling pathways are involved in sensitivity and resistance to BMPs and TGF- β

Research over the past years has revealed the important role of the non-Smad signaling pathways for both BMP- and TGF- β -induced effects. These non-Smad pathways contribute to many of the known effects of BMPs and TGF- β , e.g. induction of apoptosis and EMT, in addition to their role in regulating the Smad pathway through e.g. Smad linker-region phosphorylation. We demonstrated constitutive active p38 MAPK in the TGF- β -sensitive cell lines (paper II), and showed that inhibition of p38 MAPK could reduce the induction of cell death by TGF- β compared to control cells. In accordance with this, other studies have shown the involvement of p-p38 in TGF- β -mediated apoptosis [161] and that p-p38 can enhance sumoylation of Smad4, which positively contributes to both TGF- β and BMP target-gene induction [162].

In paper II we detected constitutive active ERK1/2 MAPK in the TGF- β -resistant cell lines. Our data implied that constitutively active ERK1/2 might be important for resistance to TGF- β . We hypothesized that pERK1/2 would phosphorylate the linker region of Smad2, as is known from the literature, thus hindering pSmad2 nuclear translocation and inducing TGF- β resistance. However, we did not detect increased Smad2 linker phosphorylation in the resistant cell lines compared to the sensitive cell lines. In addition, treatment with the specific MEK inhibitor (UO126) did not sensitize the resistant cell lines to TGF- β . It has been demonstrated that TGF- β -resistant hepatocytes expressed constitutive active ERK1/2 MAPK [163], and that ERK1/2 inhibition or knockdown rendered the cells sensitive to TGF- β -induced apoptosis. Furthermore, pERK1/2 impaired the upregulation of NOX4, an NADPH oxidase vital for the mitochondrial-dependent apoptosis. Our study indicated that ERK1/2 MAPK is not involved in the mechanism behind TGF- β resistance in B-cell lymphoma cell lines. However, we cannot completely reject our hypothesis, as the inhibitor used did not fully block pERK activation, and we did not test ERK1/2 knockdown by siRNA treatment.

BMP-2 was demonstrated to induce proliferation of ROS-50 cells (paper I) and TGF- β induced proliferation of Raji cells (paper II). Raji represents an EBV-positive BL cell line, and EBV-positive BLs have previously been demonstrated to be either refractory to TGF- β growth

inhibition, or to react by growth stimulation [131]. The EBV-protein latent membrane protein 1 (LMP-1) has been implicated to be responsible for the altered TGF- β response, as knockdown of LMP-1 rendered the EBV-positive B cells sensitive to TGF- β [164]. In addition, p38 MAPK inhibition led to increased apoptosis after TGF- β stimulation of Raji cells, indicating that p38 is activated by LMP-1 and protects against apoptosis [165]. This is contradictory to the suggested role of p38 in TGF- β -sensitive cell lines, presented in paper II and by others [161], where p38 is linked to the apoptotic effects of TGF- β in Ramos (paper II) and BL-41 [161]. These cell lines are also of BL origin, but they are not EBV-positive BL. BMP-2 has been shown to induce proliferation of different cell types, including prostate cancer and non-small cell lung carcinoma (NSCLC); the latter result obtained *in vivo* [166;167]. Langenfeld and colleagues reported that BMP-2 induced growth stimulation in an NSCLC cell line, albeit only transiently *in vitro* and in the presence of 5% FCS [167]. These effects were possibly due to upregulation of Ras-ERK MAPK or other contextual influences. Under serum-free conditions, BMP-2 stimulation decreased cell growth by 50%. Taken together, non-Smad signaling pathways are important for the effects of BMPs and TGF- β in B-cell lymphoma. In addition, constitutive active p38 MAPK is involved in the sensitivity to TGF- β in B-cell lymphoma.

4.3 ID1 is a common target gene for BMPs and TGF- β

The Id proteins are HLH proteins, which inhibit other basic HLH proteins (see chapter 1.3.1.4). BMPs and TGF- β are known to regulate the expression of ID1 [70]. BMPs have been demonstrated to upregulate ID1 in several cell types, whereas for TGF- β , downregulation of ID1 has been demonstrated in many cell types [168]. Kang and colleagues reported that all ID genes were downregulated in epithelial cell lines upon TGF- β stimulation, as seen by a microarray study. The repression was demonstrated to be mediated by Activating transcription factor 3 (ATF3), together with Smad3. ATF3 can be upregulated by TGF- β . However, several papers have reported induction of ID1 upon TGF- β stimulation [169;170], and we have demonstrated Id-1 protein upregulation after TGF- β stimulation in both paper II and III, in B-cell lymphoma cell lines as well as in an epithelial cell line (HeLa). Upregulation of Id-1 by TGF- β might be facilitated via Smad1/5, as we only detected upregulation of Id-1 in cell lines where TGF- β induced phosphorylation of Smad1/5 in addition to Smad2. ATF3 is capable of binding Smad3,

but not Smad1 [168]. However, Liang and colleagues demonstrated that Smad3 is responsible for upregulation of ID1 by TGF- β in a human mammary epithelial cell line [170].

Id-1 is described as a negative regulator of differentiation and, interestingly, a positive regulator of proliferation [70]. It was demonstrated to be induced upon growth-factor stimulation of mouse 3T3 cells [171], and to be required for the G0- to S-phase transition in human fibroblasts, as knockdown inhibited DNA synthesis in these cells [172]. Important is the Id-induced suppression of p16, a negative regulator of the cell cycle, known to induce senescence [173;174]. The suppression of p16 is probably mediated by direct physical interaction with and repression of the transcription factor Ets2. In addition, knockdown of ID1 in several other cell types, e.g. lung and prostate cancer, leads to growth suppression [175;176]. However, Sivertsen and colleagues demonstrated that ID1 knockdown abrogated the growth-inhibitory function of BMP-6 in Jurkat TAg cells [106], and others have also demonstrated that Id-1 and other Id proteins are important for growth inhibition [177-179]. We did not knock down ID1 to test whether it is essential for the growth inhibition induced by BMPs and TGF- β , as seen in paper I and II. However, in paper II, Id-1 was only upregulated in TGF- β -sensitive cell lines, indicating that Id-1 could be one possible mediator of growth inhibition in these cells. Nevertheless, Id proteins mediate pro-proliferative effects in most cell types, and are implicated as a target for therapy in many cancer types [180]. Interestingly, the studies showing that Id proteins can have growth-inhibitory effects are all conducted on cells of hematological origin, suggesting that Id-induced effects are cell-type dependent. In addition, as Id-1 is induced by different stimuli; both growth-inducing and growth-inhibiting cytokines, this might determine the outcome of Id-1 induction. Possibly, growth factors inducing Id-1 would lead to pro-proliferative effects, whereas growth inhibitors inducing Id-1 would lead to the contrary. This could be explained by the basic HLH transcription factors available that Id-1 can bind to. In this respect, Pillai and colleagues evidenced that Id-1 facilitates the growth and metastasis of NSCLC in response to EGF [175], and Sivertsen and colleagues demonstrated that Id-1 facilitates the growth-inhibiting effects of BMP-6 [106]. In addition, Kee and colleagues showed that Id-3 was responsible for the growth inhibition of TGF- β on B-lymphocyte progenitor cells [178]. Taken together, Id-1 induces both pro-proliferative and anti-proliferative effects, and this is probably cell-type and context dependent.

4.4 The role of receptor internalization and adaptor proteins

Recent investigations have shown that TGF- β and BMP receptors are internalized in the absence of ligand, but also after binding of ligand, and this might be of importance for the signaling. Hartung and colleagues reported that BMP-induced Smad1/5 phosphorylation is not dependent on internalization via clathrin-coated pits, but that induction of the BMP-responsive element (BRE)-luc reporter construct was suppressed upon inhibition of internalization [181]. This suggests that internalization via clathrin-coated pits is important for the biological effects of BMPs. Internalization of Alk-6 has been shown to depend on T β RIII and β -arrestin2, which was not the case for Alk-3 [182]. This demonstrates that the different BMP receptors are regulated in specific manners, which can influence the functional outcome of the signaling. Regarding TGF- β , several studies have shown the importance of internalization for signaling [48;49;183;184], whereas others demonstrated that internalization is not required [47;185]. Doré and colleagues reported that the mechanisms of receptor endocytosis differ between fibroblasts and epithelial cells [186], thus raising the possibility that internalization requirements differ between cells of different origin. More research on this area is needed to understand the mechanisms of internalization; whether this is an essential part of the receptor signaling and whether this is differently regulated in different cell types. Compared to other signaling pathways, e.g. EGFR, where internalization is dependent on and regulated by ligand binding, internalization of TGF- β superfamily receptors might be differently regulated.

In paper III we aimed to investigate the role of SARA in TGF- β signaling in B-cell lymphoma cell lines, as SARA has been proposed as an essential adaptor protein in this signaling pathway [75]. We did not see any correlation between the SARA expression level and the level of TGF- β -induced phosphorylation of Smad2 or Smad1/5 in B-cell lymphoma cell lines. Due to this observation we decided to further elucidate the role of SARA. As B-cell lymphoma cell lines are difficult to manipulate, we performed the mechanistical studies in HeLa cells. We succeeded to efficiently downregulate SARA with siRNA. Quantification of Western blots demonstrated that approximately only 1% SARA protein was left in the siRNA-treated cells compared to cells treated with non-coding siRNA (unpublished data). We found no indications that SARA is essential for TGF- β signaling in B-cell lymphoma cell lines and HeLa cells (paper III). This is in

contrast to the conclusions drawn by other groups, although the literature is not in agreement considering the specific role of SARA [47;75;187]. Generally, it has been proposed that SARA is localized to the early endosomes and can bind to TGF- β type I and type II receptors, Smad2 and Smad4, and therefore that SARA is essential for facilitating Smad phosphorylation. However, many of the studies have used overexpression of either wild-type (WT) or mutated versions of SARA. Of importance, overexpression of FYVE domain proteins has led to morphological changes in endosomes. This also includes overexpression of SARA, which has been shown to induce gross enlargement of endosomes [188-190]. This will most likely affect any signaling pathway induced via early endosomes, as has been proposed for the TGF- β signaling pathway. Taken together, we believe that the conclusions drawn from these prior studies might be biased due to the effects the overexpression *per se* has on the morphology of the endosomes, and should be interpreted with caution. None of the other studies have used knockdown of SARA as a tool to study the role of SARA in TGF- β signaling. The only study where SARA knockdown has been studied was published by Runyan and colleagues [191]. In this study the role of SARA in cell adhesion was analyzed, and they proposed that SARA downregulation is important during EMT.

The fact that we do not see an essential role for SARA in TGF- β signaling in B-cell lymphoma or HeLa cells could be due to redundancy. Homologous proteins could rescue the role of SARA in the signaling pathway. Therefore, in addition to knocking down SARA, we also knocked down Endofin to investigate whether Endofin could rescue the role of SARA in TGF- β signaling (paper III). Endofin is another FYVE domain-containing protein that has approximately 50% homology with SARA and has been suggested to have a role in BMP signaling. We demonstrated that Endofin could not rescue the role of SARA as TGF- β signaling was not influenced by Endofin knockdown, either alone or in combination with SARA knockdown (paper III). However, we cannot rule out that other adaptor proteins could rescue SARA upon knockdown, as we only tested Endofin for this purpose. A potential candidate might be Hrs, an essential component of the endosomal sorting complex required for transport (ESCRT) machinery, as Hrs was demonstrated to cooperate with SARA in recruiting Smad2/3 to the activin receptor complex in mouse embryonic cells [192]. Endofin has been proposed as an adaptor protein for BMP-mediated Smad phosphorylation [100]. We did, to date, not examine the role of Endofin in BMP

signaling in B-cell lymphoma. Overexpression of Endofin has also been shown to induce morphological changes of early endosomes [189].

SARA expression or its role in a cancerous setting has, to our knowledge, never been explored. Regarding cells of the immune system, one study has been conducted on CD4⁺ T cells [193]. Kunzmann and colleagues have shown that SARA expression is induced upon TGF- β stimulation, and that SARA WT overexpression attenuated the effects of TGF- β on activated T cells, although activated T cells respond stronger to TGF- β compared to naive T cells. They proposed that SARA is a negative regulator of TGF- β signaling. We did not see enhanced expression of SARA protein in B-cell lymphoma cell lines upon TGF- β stimulation (paper III).

Adaptor proteins other than SARA and Endofin, which can influence TGF- β signaling, have also been described. Cytoplasmic promyelocytic leukaemia (cPML) tumor suppressor was demonstrated to mediate the potential interaction between SARA and R-Smads, and could also bind TGF- β type I and II receptors [194]. In addition, PML competitor for TG-interaction factor (TGIF)-association (PCTA) was proposed to further induce the effects of TGF- β by binding to TGIF [195]. TGIF is a repressor of TGF- β signaling in the nucleus. It is known to bind cPML in the nucleus, thereby preventing Smad2 phosphorylation [196]. We did not investigate the role of these adaptor proteins in B-cell lymphoma.

4.5 Epidermal growth factor receptor sorting and degradation

Upon binding of EGF to its receptors, the whole complex is internalized to early endosomes and further sorted either for recycling or for degradation, the latter being the common pathway [138]. In the sorting process several adaptor proteins play an important role, e.g. the ESCRT machinery [197;198]. In our study we have demonstrated that SARA, which localizes to early endosomes, has a role in the sorting and degradation of EGFR (paper III). Knockdown of SARA led to reduced EGFR internalization and degradation. It is likely that the different adaptor proteins present are located at different stages in the sorting process and play slightly different roles, as is known for Hrs, Vps22 and Vps24 [199]. This leads to different effects upon knockdown. Raiborg

and colleagues demonstrated that neither Hrs nor other ESCRT subunits were required for endocytosis of EGFR; however, depletion of Hrs or Tsg101 led to enhanced recycling of EGFR whereas depletion of Vps22 and Vps24 did not [199]. Knockdown of SARA did not influence the EGFR recycling rate (unpublished data). Moreover, knockdown of Hrs and other ESCRT subunits strongly inhibited EGFR degradation [199]. Endofin has recently been implicated to have a role in EGFR signaling from endosomes [200]. Toy and colleagues demonstrated that mislocalization of Endofin through mutations in the FYVE domain led to enhanced pERK signaling. They did not investigate whether Endofin has a role in EGFR sorting and degradation. However, since knockdown of Endofin did not affect Rh-EGF uptake to the same extent as SARA knockdown (paper III), this is unlikely. On the other hand, SARA knockdown could possibly influence EGFR signaling, as we see effects on sorting and degradation. This needs to be further investigated. Due to the fact that both SARA and Endofin have been proposed roles in the EGFR sorting or signaling, respectively, in addition to their proposed roles in TGF- β and BMP signaling, they might possibly be involved in several trafficking and signaling pathways induced from early endosomes. In this regard, Coumailleau and colleagues have reported a role of SARA endosomes in Delta and Notch trafficking during asymmetric cell division in *Drosophila* development [201].

4.6 TGF- β and BMP signaling pathways as targets for therapies

Many studies, including paper I and II in this study, demonstrate the resistance to both BMP and TGF- β growth-inhibitory effects that is developed in cancers of highly different origin. In addition, these cytokines can act as tumor promoters. This is a rationale for developing therapies directed against the cytokines themselves or their signaling pathways. Initially, it was thought that TGF- β in itself could be used as a treatment for cancer, considering its growth-inhibitory features [202]. However, as the knowledge of TGF- β 's tumor-promoting role in cancer settings increased, it became clear that inhibitors of the TGF- β signaling pathway could be a better way to treat cancer. Nevertheless, the dual role of TGF- β led to the rise of skepticism towards inhibition of the TGF- β signaling pathway in cancer patients, as it was thought that this could fuel up under neoplastic cells by blocking the suppressive effects of TGF- β on normal and early-staged cancerous cells. However, research showed the value of inhibiting the TGF- β pathway in cancer,

and to date several compounds are investigated either in preclinical or clinical trials [203;204]. Antisense oligonucleotides, antibodies, peptide aptamers, receptor kinase inhibitors and soluble TGF- β receptors are among the therapies that currently are in development. Some of them have shown promising results, e.g. antisense oligonucleotides against TGF- β 2 or antibodies against different TGF- β isoforms, and are now tested in phase II- or III-studies in patients suffering from pancreatic cancer, high-grade glioma and other cancer types. Many of these compounds can be used in combination with conventional chemotherapy [202]. Surprisingly, very little side-effects have been unraveled during the conducted studies [203]. Although knockdown of TGF- β receptors in mouse models led to inflammation, these toxic effects have not been observed in patients. This is most likely due to the fact that the inhibitors are not capable of totally blocking the signaling pathway, and the time frame in which the inhibitors are used is relatively short. Therapies targeting BMPs or BMP signaling have been implicated to have potential in cancers that primarily metastasize to bone, e.g. prostate or breast cancer [205;206]. None of the therapies under development have, to our knowledge, been tested on hematologic malignancies. Naka and colleagues implicated that TGF- β inhibitors could be used in the therapy of chronic myeloid leukemia (CML) [207]. Both *in vitro* and *in vivo* data demonstrated that blocking the TGF- β pathway led to less leukaemia-initiating cells. In conclusion, because TGF- β can act both as a tumor suppressor and as a tumor promoter, the usage of TGF- β inhibitors in hematologic malignancies needs to be carefully considered.

5. Conclusion

In this study we have elucidated important aspects of the BMP and TGF- β signaling pathways in B-cell lymphoma. We have demonstrated that several B-cell lymphoma cell lines have developed resistance to BMPs and TGF- β , and that the sensitivity to the antiproliferative effects correlates with Smad1/5 signaling. Furthermore, we have revealed that the expression of BMP and TGF- β receptors are not reduced and therefore cannot be the mechanism behind the BMP and TGF- β resistances in B-cell lymphoma. However, Smad7 upregulation is a potential mechanism behind the resistances. In addition, we have demonstrated that SARA is not an essential part of the TGF- β signaling pathway in B-cell lymphoma or HeLa cells. In more detail this study demonstrates that:

- The mechanisms behind resistance to BMPs in B-cell lymphoma are most likely situated early in the pathway, as the resistance correlates with lower levels of Smad1/5/8 signaling. The receptors are not downregulated in the resistant cell lines; however, we cannot rule out that mutations are present that render the receptors unable to induce signaling but do not interfere with the cell-surface expression.
- Overexpression of Smad7 rendered the SUDHL-6 cells resistant to BMPs, thus, upregulation of Smad7 is a potential mechanism behind resistance in B-cell lymphoma.
- TGF- β signals through Smad2 in both resistant and sensitive B-cell lymphoma cell lines, hence, Smad2 phosphorylation alone cannot be essential for the antiproliferative effects of TGF- β in B-cell lymphoma. We propose that phosphorylated Smad1/5 is important for the antiproliferative effects of TGF- β demonstrated in certain B-cell lymphoma cell lines.
- Constitutively active p38 MAPK is, at least in part, important for the sensitivity to TGF- β in B-cell lymphoma cell lines.
- The adaptor protein SARA is not essential for the TGF- β signaling pathway, as almost complete knockdown did not affect the signaling at any level. Endofin, another FYVE domain protein, does not rescue SARA upon SARA knockdown.
- We propose a more general role for SARA in regulating the sorting of receptors via the early endosomes. Importantly, SARA is involved in the sorting of the EGFR, and SARA knockdown affects receptor degradation.

6. Future studies

In general, further studies are needed to elucidate the mechanisms behind the resistances to BMPs and TGF- β in B-cell lymphoma. However, as discussed in chapter 4.1.2, genetic manipulations of B-cell lymphoma cell lines or their normal counterparts have proven difficult. The establishment of retroviral transduction in our lab may nevertheless render it possible to conduct some of our planned experiments in the near future. Knockdown of receptors, Smad1/5 or Id-1 in the TGF- β -sensitive cell lines would provide evidence whether these proteins are important for the antiproliferative effects of TGF- β . Other possible mechanisms that we have not investigated are the presence of mutations in receptors or downstream signaling components or the role of other adaptor proteins, phosphatases or miRNAs. In addition to *in vitro* studies, animal models could be feasible to see the physiological importance of e.g. TGF- β -mediated signaling via Smad1/5 or the role of SARA in TGF- β signaling and other signaling pathways.

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RESEARCH ARTICLE

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TGF- β -induced growth inhibition in B-cell lymphoma correlates with Smad1/5 signalling and constitutively active p38 MAPK

Maren Bakkebø^{1,2}, Kanutte Huse^{1,2}, Vera I Hilden^{1,2}, Erlend B Smeland^{1,2}, Morten P Oksvold^{1,2*}

Abstract

Background: Cytokines of the transforming growth factor β (TGF- β) superfamily exert effects on proliferation, apoptosis and differentiation in various cell types. Cancer cells frequently acquire resistance to the anti-proliferative signals of TGF- β , which can be due to mutations in proteins of the signalling cascade. We compared the TGF- β -related signalling properties in B-cell lymphoma cell lines that were sensitive or resistant to TGF- β -induced anti-proliferative effects.

Results: TGF- β sensitive cell lines expressed higher cell surface levels of the activin receptor-like kinase 5 (Alk-5), a TGF- β receptor type 1. The expression levels of the other TGF- β and bone morphogenetic protein receptors were comparable in the different cell lines. TGF- β -induced phosphorylation of Smad2 was similar in TGF- β sensitive and resistant cell lines. In contrast, activation of Smad1/5 was restricted to cells that were sensitive to growth inhibition by TGF- β . Moreover, with activin A we detected limited anti-proliferative effects, strong phosphorylation of Smad2, but no Smad1/5 phosphorylation. Up-regulation of the TGF- β target genes *Id1* and *Pai-1* was identified in the TGF- β sensitive cell lines. Constitutive phosphorylation of MAPK p38 was restricted to the TGF- β sensitive cell lines. Inhibition of p38 MAPK led to reduced sensitivity to TGF- β .

Conclusions: We suggest that phosphorylation of Smad1/5 is important for the anti-proliferative effects of TGF- β in B-cell lymphoma. Alk-5 was highly expressed in the sensitive cell lines, and might be important for signalling through Smad1/5. Our results indicate a role for p38 MAPK in the regulation of TGF- β -induced anti-proliferative effects.

Background

The members of the TGF- β superfamily of cytokines, which consists of TGF- β s, bone morphogenetic proteins (BMPs) and activins, exert potent effects on proliferation, apoptosis and differentiation on many different cell types, including primary B cells [1,2]. The signalling is initiated through heterotetrameric complexes of type I and type II receptors. The cytokines bind to a type II receptor, and type I is recruited and activated through phosphorylation. There are five type II and seven type I receptors which form complexes with the TGF- β superfamily of cytokines. TGF- β induces signalling through TGF- β receptor type II (T β RII) and Alk-5 (type I),

whereas activin A and B induce signalling through activin receptor type II (ActRII), activin receptor type II b (ActRIIb), Alk-4 and Alk-7 (type I) [3]. The intracellular receptor regulated Smad proteins (R-Smads) are phosphorylated by the type I receptors. Smad2 and 3 are the main R-Smads involved in TGF- β and activin signalling [4]; although several recent reports have shown that TGF- β can induce Smad1/5/8 signalling as well [5,6]. BMPs activate Smad1/5/8. R-Smads interact with the common Smad, Smad4, and translocate to the nucleus, where the complex, together with other transcription factors, regulates gene expression of e.g. *Pai-1*. *Pai-1* plays an important role throughout many cell systems, and is involved in cell motility, angiogenesis and cancer progression [7] in addition to anti-proliferative activity [8]. It has been shown that inhibitory Smads, Smad6 and 7, inhibit the pathway at several levels,

* Correspondence: moroks@rr-research.no

¹Dep. of Immunology, Institute for Cancer Research, Oslo University Hospital Montebello, Oslo, Norway

Full list of author information is available at the end of the article

i.e. interaction between R-Smad and receptor or between R-Smads and Smad4 [3]. There is extensive crosstalk with other signalling pathways, such as p38, ERK1/2, JNK, PI3K and Wnt [9]. It is suggested that this regulation often occurs through phosphorylation of the linker region of R-Smads, which can be activating or inhibitory to the effects of TGF- β s, activins or BMPs.

In cancer, TGF- β frequently loses its anti-proliferative effects, and sometimes gains pro-proliferative features, often associated with epithelial-to-mesenchymal-transition and metastasis of epithelial cells. Loss of anti-proliferative effects can be due to mutations, gene silencing or over-expression of inhibitors [10,11]. In lymphoma and other haematological malignancies, aberrant expression of receptors and mutations in Smads have been found, although the reported frequencies of aberrations involving the TGF- β pathway in lymphoma are lower than in many other cancer types [12,13]. For example, down-regulation of T β RII RNA has been demonstrated in Burkitt lymphoma (BL) cell lines which express the full range of latent EBV genes [14].

Our aim was to elucidate the effects of TGF- β and activin A on lymphoma cell lines, to study the signalling pathways involved and to look for possible mechanisms behind sensitivity or resistance to these cytokines. We suggest that signalling through Smad1/5 can be important for maintaining sensitivity to TGF- β growth inhibitory effects. In addition, constitutively active p38 MAPK indicates a role for this kinase in the regulation of TGF- β -induced anti-proliferative effects.

Results

B-cell lymphoma show reduced sensitivity to TGF- β compared to primary B cells

Many cancer types develop resistance to TGF- β -induced growth inhibition. We tested the anti-proliferative effects of TGF- β on 11 different B-cell lymphoma cell lines, and compared these results to human peripheral blood CD19⁺ B cells. For further studies on signalling we selected five of these cell lines; three of these showed high sensitivity to TGF- β treatment; although not to the same extent as primary B cells, whereas two were resistant to the growth inhibiting effects of TGF- β (Figure 1A). In line with previously published data, TGF- β treatment of primary B cells inhibited proliferation by 85% compared to non-treated control B cells (Figure 1A). More data on additional cell lines are included in Additional file 1, Fig. S1 (two sensitive cell lines, Oci-Ly 3 and Oci-Ly 10, and one resistant cell line, Raji).

In addition to TGF- β , we tested the anti-proliferative effects of activin A and B, and detected no major effects on proliferation by these cytokines (Figure 1B and data not shown). Primary B cells were partly inhibited by

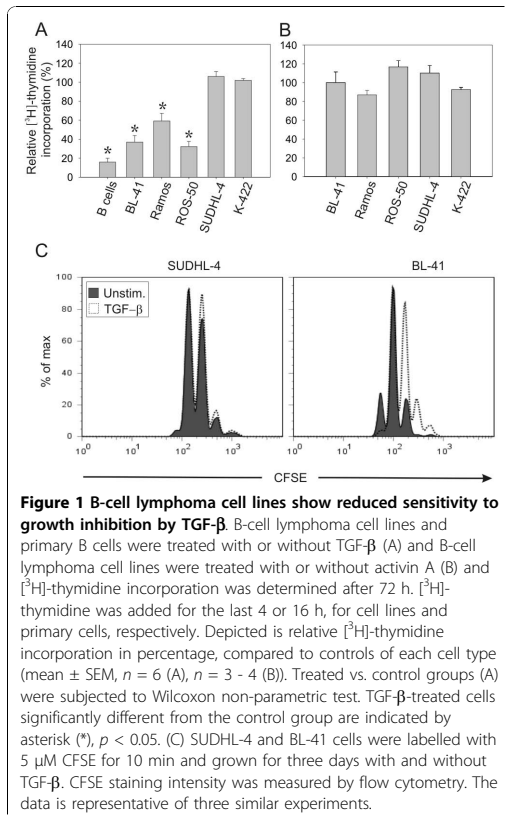


Figure 1 B-cell lymphoma cell lines show reduced sensitivity to growth inhibition by TGF- β . B-cell lymphoma cell lines and primary B cells were treated with or without TGF- β (A) and B-cell lymphoma cell lines were treated with or without activin A (B) and [³H]-thymidine incorporation was determined after 72 h. [³H]-thymidine was added for the last 4 or 16 h, for cell lines and primary cells, respectively. Depicted is relative [³H]-thymidine incorporation in percentage, compared to controls of each cell type (mean \pm SEM, $n = 6$ (A), $n = 3 - 4$ (B)). Treated vs. control groups (A) were subjected to Wilcoxon non-parametric test. TGF- β -treated cells significantly different from the control group are indicated by asterisk (*), $p < 0.05$. (C) SUDHL-4 and BL-41 cells were labelled with 5 μ M CFSE for 10 min and grown for three days with and without TGF- β . CFSE staining intensity was measured by flow cytometry. The data is representative of three similar experiments.

activin A, with a mean inhibition of 34% ($n = 3$, data not shown).

Additionally, we measured cell division to compare the effects of TGF- β in sensitive and resistant cells. In the resistant SUDHL-4 cells no inhibition of cell division was detected. In contrast, TGF- β induced a clear inhibition in BL-41 cells after three days, as evidenced by the CFSE histograms (Figure 1C).

TGF- β sensitive cell lines express high cell surface levels of Alk-5

To determine the role of the different TGF- β receptors during Smad signalling in B-cell lymphoma, we measured endogenous cell surface levels of the receptors Alk-1, Alk-5 and T β RII by flow cytometry on lymphoma cell lines and primary B cells. The TGF- β sensitive cell lines expressed higher levels of Alk-5 compared to the resistant cell lines and primary B cells (Figure 2A and 2B). The specificity of the anti-Alk-5 antibody was tested by blocking with the peptide used for immunization before

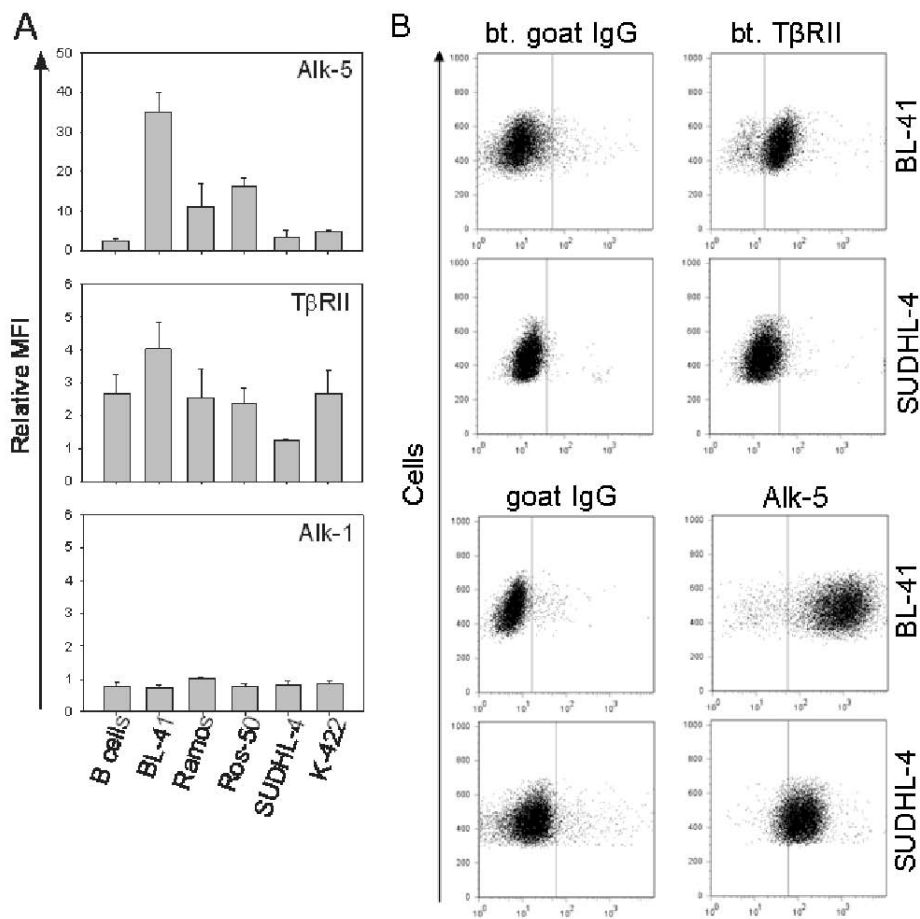


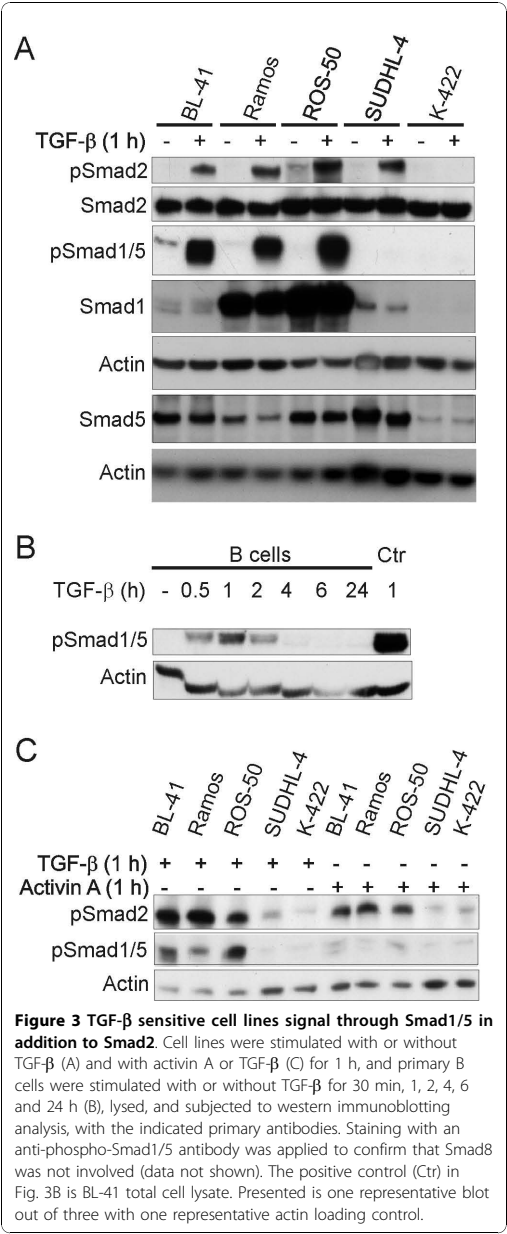
Figure 2 Expression of cell surface TGF- β receptors. Endogenous levels of the receptors Alk-5, T β RII and Alk-1 were determined by flow cytometry. Illustrated is (A) median fluorescence intensity relative to irrelevant control for primary B cells and five cell lines (mean \pm SEM, $n = 3$) and (B) dot plot for BL-41 and SUDHL-4 (one representative of three). For Alk-1 and T β RII, biotinylated goat IgG is used as control, for Alk-5, goat IgG is used as control.

flow cytometry (data not shown). T β RII was expressed in all cell lines tested and in primary B cells, with no striking differences between TGF- β sensitive and resistant cell lines (Figure 2A and 2B). Alk-1 was expressed at low levels (Figure 2A). Furthermore, the type I and type II activin receptors (Alk-4, Alk-7, ActRII and ActRIIb) were similarly expressed in all cell lines (data not shown). It has been shown that TGF- β can signal through the BMP-receptors Alk-2 and Alk-3 [5]. We therefore examined the expression levels of these two BMP type I receptors. Of the sensitive cell lines, only ROS-50 expressed low levels of Alk-2 and Alk-3, whereas Ramos expressed

some Alk-2 and higher levels of Alk-3 (Huse, K. *et al.*, submitted).

Activation of Smad1/5 in TGF- β sensitive cells

To investigate signalling pathways triggered by TGF- β , Western immunoblotting analysis was performed. TGF- β induced activation of the canonical Smad2 pathway in primary B cells (data not shown) and in all cell lines, except K-422 (Figure 3A, Additional file 2, Fig. S2 and Additional file 3, Fig. S3). However, we detected no major differences in levels of Smad2 phosphorylation between sensitive and resistant cell lines. Recently, there



has been focus on TGF- β signalling through Smad1/5 in addition to Smad2/3 [5,6]. Interestingly, in the sensitive cell lines as well as in primary B cells, TGF- β induced Smad1/5 phosphorylation (Figure 3A and 3B and Additional file 2, Fig. S2). Immunoblotting with anti-

pSmad1/5/8 and anti-pSmad1/5 was comparable, indicating that Smad8 is not important in TGF- β signalling in B-cell lymphoma (data not shown). Activin A, which had limited effects on proliferation, induced phosphorylation of Smad2 only in the TGF- β sensitive cell lines. Phosphorylation of Smad1/5 was not detected after activin A treatment (Figure 3C). We examined endogenous levels of Smad1 and Smad2 proteins, and found that Ramos and ROS-50 cells expressed higher levels of Smad1 compared to the other cell lines. No major differences in Smad2 levels were observed (Figure 3A). Taken together, the data suggest that Smad1/5 is involved in controlling the anti-proliferative effects of TGF- β in B-cell lymphoma cell lines.

To check whether inhibitory Smads play a role in resistance to TGF- β , we assessed the endogenous protein levels of Smad6 and 7. However, only minor differences in expression levels were seen when comparing the different cell lines (data not shown).

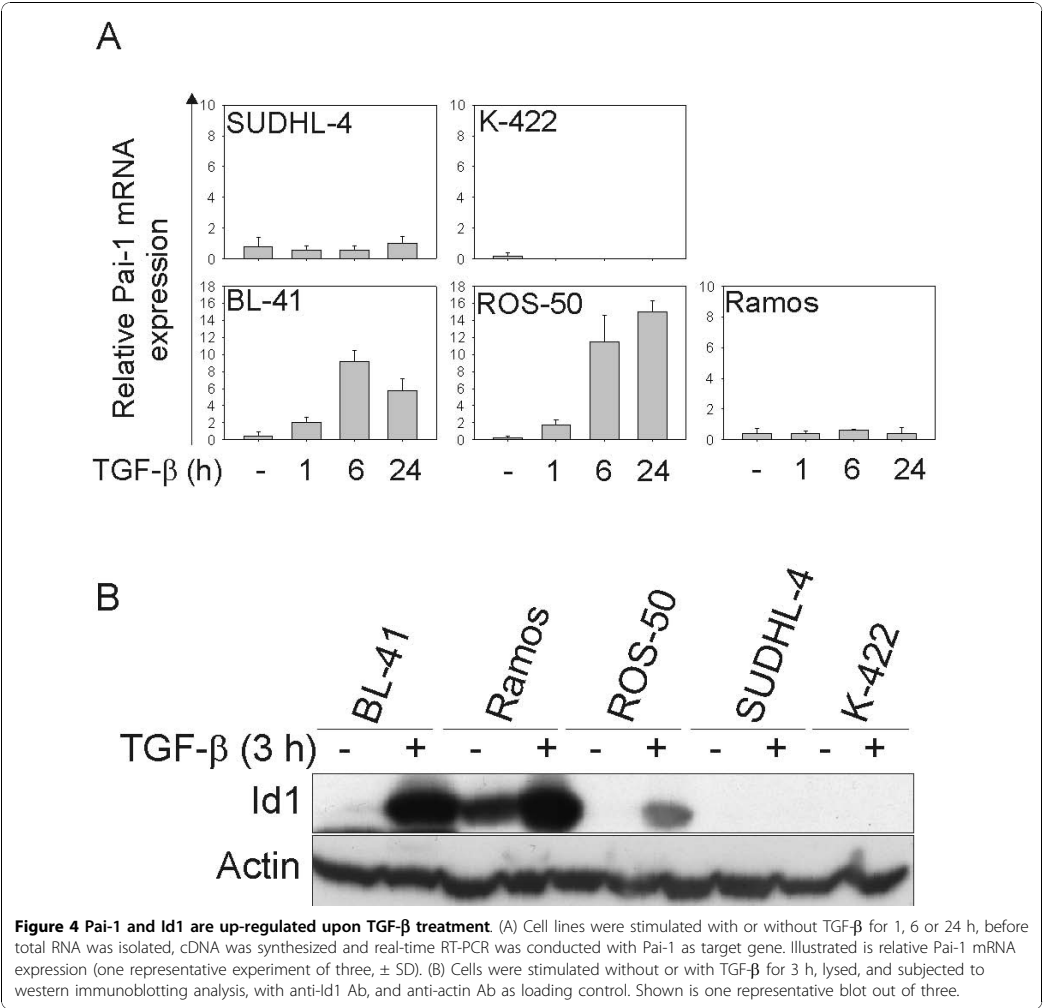
Activation of TGF- β target genes

To investigate whether the TGF- β -induced signalling continued into the nucleus and up-regulated known TGF- β target genes, we measured Pai-1 mRNA. Interestingly, TGF- β induced up-regulation of Pai-1 in two of the sensitive cell lines (Figure 4A). In addition, we demonstrated that Id1, a known BMP target gene, was induced to different degrees upon TGF- β treatment in the sensitive cell lines (Figure 4B). The resistant cell lines showed no up-regulation of either of these target genes (Figure 4A and 4B). These data imply that there are differences between TGF- β sensitive and resistant cell lines regarding induction of TGF- β target genes.

p38 MAPK is constitutive active in TGF- β sensitive cells

We further investigated other signalling pathways known to crosstalk with the canonical Smad pathway. Of interest, we found high constitutive levels of phosphorylated p38 MAPK (Thr180/Tyr182) in the TGF- β sensitive cell lines (Figure 5A and Additional file 4, Fig. S4). The resistant cell lines expressed minimal levels of active p38 MAPK compared to the sensitive cell lines. We also found high constitutive levels of phosphorylated ERK1/2 MAPK (Thr202/Tyr204) in the TGF- β resistant cell lines, but also in one of the sensitive cell lines (Figure 5A). TGF- β did not affect the level of phosphorylated ERK1/2. Screening of other activated signalling molecules, i.e. phosphorylated Akt, JNK MAPK, TAK and MKK 3/6 did not reveal any correlation to sensitivity or resistance to TGF- β (data not shown).

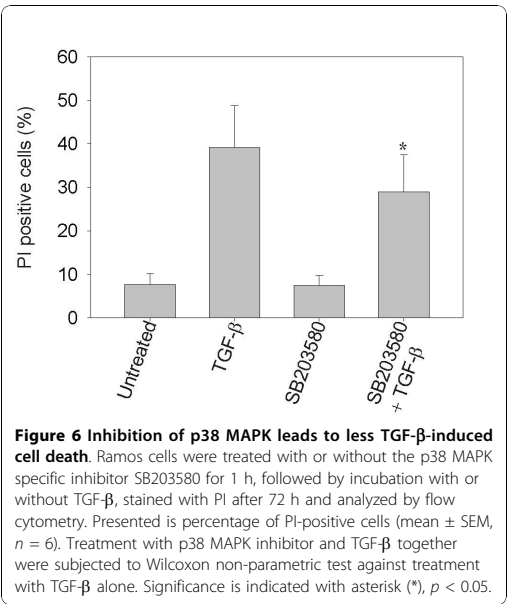
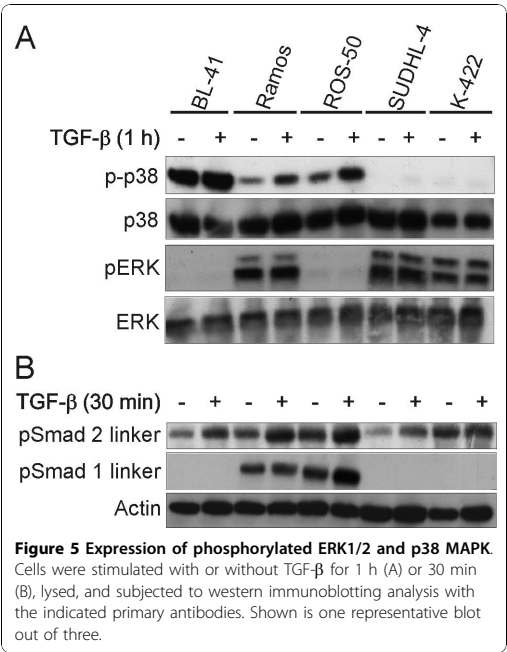
Due to high levels of activated ERK1/2 MAPK in the resistant cell lines, and the fact that this can alter the canonical Smad signalling pathway through phosphorylation of the linker region, we investigated phosphorylation



levels of the Smad2 (Ser245/Ser250/Ser255) and Smad1 (Ser206) linker regions. Smad1 linker phosphorylation was detectable in two TGF-β sensitive cell lines, and TGF-β only slightly altered the level of linker phosphorylation in these cell lines (Figure 5B). In contrast, no major differences in Smad2 linker region phosphorylation were observed between the sensitive and resistant cell lines. These results imply that activated ERK1/2 MAPK could be involved in resistance to TGF-β in B-cell lymphoma cell lines, although phosphorylation of the linker region of Smad2 seems not to be the mechanism. We suggest that activated p38 MAPK could be important for sensitivity to TGF-β.

Inhibition of p38 MAPK leads to reduced sensitivity to TGF-β

To test whether p38 contributes to TGF-β sensitivity, we used the p38-specific inhibitor SB203580 in the TGF-β sensitive cell line Ramos. When phosphorylation of p38 was inhibited, we observed reduced sensitivity to TGF-β-induced anti-proliferative effects compared to the control group (Figure 6). TGF-β induced cell death in 39% of the cells, whereas TGF-β together with SB203580 differed significantly with 29% cell death ($p < 0.05$, Figure 6). The p38 inhibitor also reduced TGF-β-induced apoptosis as determined by TUNEL analysis (data not shown). Inhibition of ERK1/2 MAPK did not



alter the effects of TGF- β on the resistant cell lines (data not shown). Thus, inhibition of p38 MAPK partially counteracts TGF- β -induced growth suppression in Ramos cells, suggesting a role for p38 MAPK in the regulation of TGF- β -induced anti-proliferative effects.

Discussion

It is known from several cancer types that TGF- β loses its anti-proliferative effects, often due to mutations in receptors or Smad proteins [15,16]. Haematological malignancies, especially B-cell lymphoma, have received less attention regarding TGF- β signalling. We sought to elucidate the effects of TGF- β on cell lines from different B-cell lymphoma subtypes, working with endogenous levels of gene expression. We found that the B-cell lymphoma cell lines examined displayed reduced sensitivity to TGF- β compared to primary B cells. This indicates that loss of sensitivity towards the growth inhibitory effects of TGF- β can be of importance for the development of B-cell lymphoma.

Although Smad2 and 3 are the main R-Smads for TGF- β signalling, we found no clear differences in TGF- β -induced Smad2 signalling when comparing sensitive and resistant cell lines. Moreover, we detected that activin A and B exerted limited anti-proliferative effects on the B-cell lymphoma cell lines, even though clear Smad2 signalling was observed in the TGF- β -sensitive cell lines upon activin A stimulation. This further indicates that Smad2 phosphorylation is not directly correlated to inhibition of proliferation. Of note, recent studies have revealed that TGF- β can also activate the Smad1/5/8 pathway. Interestingly, we observed a clear correlation between sensitivity to TGF- β and Smad1/5 phosphorylation as TGF- β induced phosphorylation of Smad1/5 in sensitive cell lines only. Smad1/5 signalling upon TGF- β treatment has to our knowledge previously not been reported in primary B cells. These data suggest that signalling through Smad1/5 is important for the functional effects of TGF- β on B-cell lymphoma cell lines of different origin. In agreement with our data, Munoz *et al.* have previously reported induction of Smad1 phosphorylation upon TGF- β treatment in follicular lymphoma cell lines and one diffuse large B-cell lymphoma cell line [17]. Moreover, they demonstrated that the functional effects of TGF- β were diminished upon treatment with Smad1 siRNA. Taken together, available data suggest that Smad1/5 is crucial for the anti-proliferative effects of TGF- β .

We found that sensitive cell lines showed higher endogenous Alk-5 levels and this expression correlated to Smad1/5 activation, as it was highly expressed in the cell lines where TGF- β induced phosphorylation of

Smad1/5. Similar results have been found in other cell systems [6]. Data by Wrighton *et al.* suggest that Alk-5 has the ability to phosphorylate Smad1, and that Smad1 can co-precipitate Alk-5 in HEK293T cells. In other cell systems, additional receptors have been demonstrated to be necessary. Daly *et al.* proved that T β RII and Alk-5 were required, but not sufficient for Smad1/5 phosphorylation [5]. They found that Alk-2 or Alk-3 can co-precipitate with T β RII and Alk-5, and that forming of the receptor complexes is dependent on cell type. Among the cell lines which induced Smad1/5 signalling, only Ramos expressed some Alk-2 and higher levels of Alk-3. Alk-1 was expressed at such low levels that it is unlikely to be involved. This was expected, because Alk-1 is believed to be present only in endothelial cells [18,19]. T β RII is most likely involved in Smad2 and Smad1/5 signalling in our cell lines, as it is the only known type II receptor for TGF- β [3]. However, the T β RII expression level differed in both sensitive and resistant cell lines. Smad2 signalling upon activin A stimulation is detected in Ramos, ROS-50 and BL-41 cells. Abrogated Smad2 signalling in the other cell lines is most likely not due to reduced expression of receptors, as we detected nearly equal expression of all known activin receptors in our cell lines. Thus, Alk-5 might be the receptor which is crucial for Smad1/5 signalling and TGF- β -induced anti-proliferative effects.

Previous work has shown a correlation between activated p38 MAPK and the apoptotic effects of TGF- β in BL-41 cells [20]. In accordance with this study, we found that p38 was constitutively phosphorylated in cell lines sensitive to growth inhibition by TGF- β . In contrast, TGF- β resistant cell lines expressed high levels of phosphorylated ERK1/2 MAPK. We successfully inhibited p38 in Ramos cells, and showed that the anti-apoptotic effects of TGF- β is dependent, at least to some degree, on the activity of p38. It is possible that p38-induced sumoylation of Smad4, which enhances TGF- β and BMP target gene activation, could explain the positive effect of phosphorylated p38 on TGF- β growth inhibition, as suggested by Ohshima *et al.* [21]. Possibly, one needs to induce ERK1/2 in addition to inhibiting p38 to diminish the effects of TGF- β . Interestingly, we detected phosphorylated ERK1/2 in Ramos cells, whereas in BL-41 and ROS-50 cells this phosphorylation was not seen. This might explain why the effects of TGF- β were reduced only in Ramos cells and not in BL-41 and ROS-50 cells (data not shown) upon adding the p38 inhibitor. Phosphorylation of the R-Smad linker region may inhibit translocation of activated Smad-complexes to the nucleus. It is demonstrated that ERK1/2 phosphorylates the linker region of Smad1 and Smad2, and this can inhibit signal transduction and the anti-proliferative effects of TGF- β [22,23]. However, the

consequences of linker-phosphorylation remain controversial [24], and we did not detect any higher levels of phosphorylation of the Smad2 linker region in TGF- β resistant compared to sensitive cell lines. The Smad1 linker region was phosphorylated in Ramos and ROS-50 cells, and this might even induce Smad1/5 signalling by TGF- β in these cells.

Id1 is a known BMP-responsive gene, which is up-regulated upon Smad1/5/8 signalling [25,26]. However, TGF- β -induction of Id proteins has previously been found in a BL cell line, CA46 [27], although it was not investigated whether Smad1/5 signalling was involved. We demonstrate induction of Id1 protein in the TGF- β sensitive cell lines (BL-41, Ramos and ROS-50) after 3 h of TGF- β stimulation. Opposed to that, Daly *et al.* did not detect induction of a luciferase reporter containing two repeats of a BMP response element in cell types where TGF- β also signals through Smad1/5 [5]. Induction of Id1 is possibly dependent on the cell type. It has been reported that TGF- β represses Id expression in epithelial cells [28].

Conclusion

To summarize, three B-cell lymphoma cell lines showed sensitivity to the TGF- β anti-proliferative effects. Sensitivity to growth inhibition by TGF- β might depend on Smad1/5 signalling in lymphoma cell lines, which possibly initiates via Alk-5 and terminates in up-regulation of Id1 and other target genes. We suggest that the regulation of proliferation by TGF- β is at least partly dependent on activated p38 MAPK. Further knock-down studies need to be assessed to confirm this theory. In the future, therapies which can restore sensitivity of lymphoma cells to TGF- β growth control by inducing Smad1/5 signalling can be helpful in treatment of B-cell lymphoma patients.

Methods

Cell culture

BL cell lines Ramos, BL-41 and Raji, diffuse large B-cell lymphoma cell lines of germinal centre B type SUDHL-4 and of activated B cell type Oci-Ly 3 and Oci-Ly 10 and follicular lymphoma cell lines K-422 and ROS-50 were cultured in RPMI (PAA Laboratories, Austria) with 100 Units/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories) and 10% fetal calf serum (PAA Laboratories), except for the Oci Ly-cells, which were cultured in IMDM (Invitrogen, CA, USA) with 55 μ M β -Mercaptoethanol (Invitrogen), 100 Units/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories) and 10% human plasma (SeraCare Life Sciences, Inc., California, USA), at 37°C with 5% CO₂ in air. Prior to all experiments, cells were grown under serum free conditions over night in X-VIVO 15 (BioWhittaker,

Switzerland). Primary human B cells from peripheral blood were isolated using CD19⁺ Dynabeads (Invitrogen), as described by Rasmussen *et al.* [29]. Peripheral blood was provided by the Blood Bank at Ullevål University Hospital with formal agreement by the blood donors, and approval by the regional ethics committee.

Reagents

Carrier-free huTGF- β 1 (10 ng/ml) and activin A and B (10 ng/ml) were purchased from R&D Systems (MN, USA). Anti-IgM (10 μ g/ml) was obtained from Jackson Immuno Research (PA, USA). [³H]-thymidine was purchased from American Radiolabeled Chemicals (MO, USA). The following Ab were used: Anti-phospho-Smad2, -phospho-Smad1/5/8, -phospho-Smad1/5, -Smad1, -Smad2, -Smad6, -phospho-p38 MAPK, -p38 MAPK, -phospho-ERK1/2 MAPK -phospho-TAK 1, -phospho-MKK3/MKK6 and -phospho-JNK MAPK Ab (Cell Signalling Technology, MA, USA), anti-actin, -ERK MAPK and -Id1 Ab (Santa Cruz, CA, USA), anti-Smad7 Ab (Abcam, MA, USA), biotinylated anti-Alk-1, -T β RII, -ActRII and -ActRIIb Ab and anti-Alk-4 and -Alk-5 Ab (R&D systems), anti-Alk-7 Ab (Millipore, MA, USA) and HRP-coupled secondary anti-rabbit, -mouse and -goat IgG Ab (DakoCytomation, Denmark). ERK inhibitors FR180204 and UO126 and p38 inhibitor SB203580 were purchased from Calbiochem (Darmstadt, Germany). The Alk-5 peptide used for blocking of anti-Alk-5 antibody was obtained from R&D systems.

Cell proliferation assays

Cells were harvested after 72 h using an automated cell harvester (Filtermate 196, Packard Instrument Company Inc., CT, USA), and [³H]-thymidine incorporation into DNA was measured on a scintillation counter (Top-Count, Packard Instrument Company Inc.). Assays were performed in triplicates in round bottom 96-well plates, 200 μ l per well; 0.1×10^6 cells/ml for cell lines and 0.375×10^6 cells/ml for B cells. [³H]-thymidine was added 4 or 16 h before measurement, respectively.

To monitor cell death, 5 μ g/ml PI was used for analysis by flow cytometry (Becton Dickinson, FACSCalibur, NJ, USA). Cell death was measured for every functional experiment conducted.

For CFSE proliferation analysis cells were labeled with 5 μ M CFSE (Molecular Probes, OR, USA) in PBS with 0.1% BSA for 10 min at 37°C. The labeling reaction was quenched by addition of cold PBS with 20% FCS. Cells were incubated in pre-warmed X-VIVO 15 and cultured over night before cells with identical CFSE staining intensity was sorted. The CFSE sorted cells were cultured for up to 3 days with or without TGF- β . FACS analysis allowed gating on individual CFSE generations (Becton Dickinson, FACS CantoII).

Apoptosis assay

Cells were harvested after 3 days and stained with TUNEL (Roche, Switzerland) according to the manufacture's protocol. Cells were analyzed by flow cytometry (Becton Dickinson, FACSCalibur).

Western immunoblotting analysis

Cells were lysed in Tris lysis buffer, pH 6.8 (62.7 mM Tris-HCl, 10% (v/v) glycerol, 2.3% (w/v) SDS, 5% β -Mercaptoethanol, 1 \times protease inhibitor mixture (Complete Mini, Roche) and 1 \times phosphatase inhibitor mixture (PhosSTOP, Roche)). Lysates were incubated at 95°C for 10 min, cleared by centrifugation at 15700 g for 5 min and protein concentrations were determined with the BioRad (CA, USA) protein assay. Samples (30-40 μ g/lane) were run on 10% or 12% SDS-polyacrylamide gels (Pierce, IL, USA) or 10% Tris-HCl Criterion gels (BioRad) and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% non-fat dry milk or 5% BSA (Sigma-Aldrich, MO, USA) in TBST buffer pH 7.6, according to the antibody manufacturer's protocol. PVDF membranes were incubated over night at 4°C with antibody diluted in 5% non-fat dry milk or 5% BSA in TBST buffer. HRP-conjugated anti-mouse, -goat and -rabbit IgG antibodies incubated for 60 min at room temperature were used followed by detection using ECL or ECL-plus (GE Healthcare, NJ, USA).

Detection of cell surface receptor expression

Cells were blocked in 1 mg/ml γ -globulin (aggregated at 63°C, 20 min (Sigma-Aldrich)) for 10 min on ice, prior to staining with anti-Alk-1, -Alk-5 or -T β RII antibodies for 30 min at 4°C. Avidin-PE (BioRad) was used as second layer. Goat Ig and biotinylated Goat Ig were used as controls. Cells were washed in PBS. Receptor levels were detected by flow cytometry (Becton Dickinson, FACSCalibur and FACS CantoII).

Real time RT-PCR analysis

RNA was isolated using the Absolutely RNA Miniprep kit (Stratagene, CA, USA) following the manufacturer's instructions. RNA concentration was obtained using NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA), and quality was assessed by gel electrophoresis using 1.5% agarose gel. RNA was stored at -80°C. Using the TaqMan kit (Applied Biosystems, CA, USA), cDNA was obtained from 1 μ g RNA for each sample and 400 ng RNA for the minus reverse transcriptase (-RT) negative controls. Conditions: 10 min at 25°C, 40 min at 42°C and 10 min at 95°C.

Real-time RT-PCR was conducted with TaqMan Universal PCR master mix (Applied Biosystems) with Pai-1 (Applied Biosystems) as the target gene and PGK-1 (Applied Biosystems) as the control gene. The cDNA

used assembled a total of 10 ng RNA per reaction. The samples had a total volume of 25 μ l, and were run on ABI Prism 7000 Sequence Detection System. Conditions: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative expression levels were calculated using the threshold cycle $\Delta\Delta$ Ct-method as described by the manufacturers' protocol. Instead of calibrating the samples to a control sample, samples were calibrated to the number 10.

Statistical analysis

Wilcoxon non-parametric test was used for all statistical measurements, with *p*-value less than 0.05 considered as significant. The statistical significance analysis in Figure 1A has been conducted on primary data, although the data depicted in the figure are normalized.

Additional material

Additional file 1: Fig. S1. B-cell lymphoma cell lines show reduced sensitivity to growth inhibition by TGF- β . B-cell lymphoma cell lines were treated with or without TGF- β and [3 H]-thymidine incorporation was determined after 72 h. [3 H]-thymidine was added for the last 4 h. Depicted is relative [3 H]-thymidine incorporation in percentage, compared to controls of each cell type (mean \pm SEM, *n* = 6).

Additional file 2: Fig. S2. TGF- β sensitive cell lines signal through Smad1/5 in addition to Smad2. Cell lines were stimulated with or without TGF- β for 1 h, lysed, and subjected to western immunoblotting analysis, with the indicated primary antibodies. Presented is one representative blot out of three. Actin was used as loading control.

Additional file 3: Fig. S3. Both TGF- β sensitive and resistant cell lines signal through Smad2. The volume of each band from Western immunoblots with pSmad2 and actin antibodies was calculated using Quantity One Analysis Software to quantify the phosphorylation of Smad2 upon TGF- β stimulation. The measured values were normalized against actin and the relative expression in TGF- β -treated BL-41 cells. Shown is relative pSmad2 expression in both sensitive and resistant cell lines, *n* = 6 (mean \pm SEM).

Additional file 4: Fig. S4. Sensitive cell lines express activated p-p38. Cells were stimulated with or without TGF- β for 1 h, lysed and subjected to western immunoblotting analysis with p-p38 and actin as primary antibodies. Shown is one representative blot out of three, and one representative actin control.

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Author details

¹Dep. of Immunology, Institute for Cancer Research, Oslo University Hospital Montebello, Oslo, Norway. ²Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway.

Authors' contributions

MB participated in the design, conducted most of the experiments, and drafted the manuscript. KH conducted some of the initial experiments. VH participated in some of the experiments. EBS participated in the study

design, and obtained funding. MPO developed the project idea, participated in the study design, coordinated and conducted some of the experiments. All the authors have critically read, commented and approved the final manuscript.

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Additional files to paper II

Additional file 1, Fig. S1.

Title: B-cell lymphoma cell lines show reduced sensitivity to growth inhibition by TGF- β .

Description: B-cell lymphoma cell lines were treated with or without TGF- β and [^3H]-thymidine incorporation was determined after 72 h. [^3H]-thymidine was added for the last 4 h. Depicted is relative [^3H]-thymidine incorporation in percentage, compared to controls of each cell type (mean \pm SEM, $n = 6$).

Additional file 2, Fig. S2.

Title: TGF- β sensitive cell lines signal through Smad1/5 in addition to Smad2.

Description: Cell lines were stimulated with or without TGF- β for 1 h, lysed, and subjected to western immunoblotting analysis, with the indicated primary antibodies. Presented is one representative blot out of three. Actin was used as loading control.

Additional file 3, Fig. S3.

Title: Both TGF- β sensitive and resistant cell lines signal through Smad2.

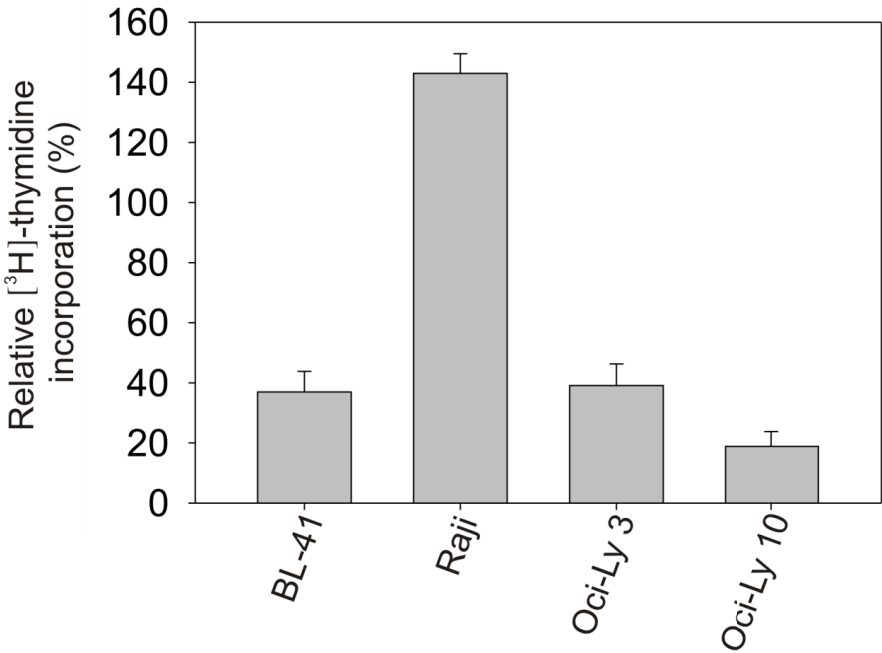
Description: The volume of each band from Western immunoblots with pSmad2 and actin antibodies was calculated using Quantity One Analysis Software to quantify the phosphorylation of Smad2 upon TGF- β stimulation. The measured values were normalized against actin and the relative expression in TGF- β -treated BL-41 cells. Shown is relative pSmad2 expression in both sensitive and resistant cell lines, $n = 6$ (mean \pm SEM).

Additional file 4, Fig. S4.

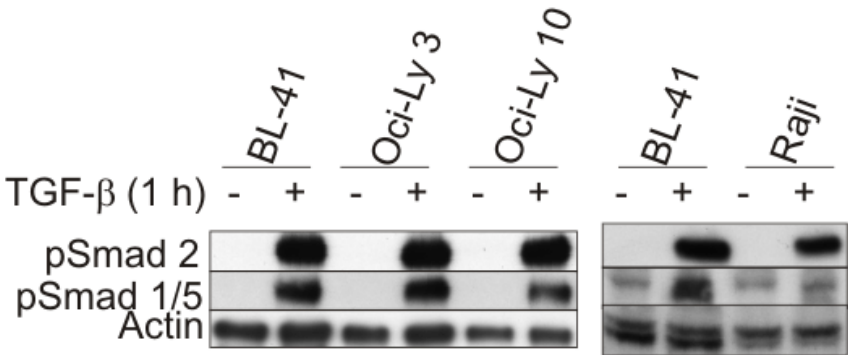
Title: Sensitive cell lines express activated p-p38.

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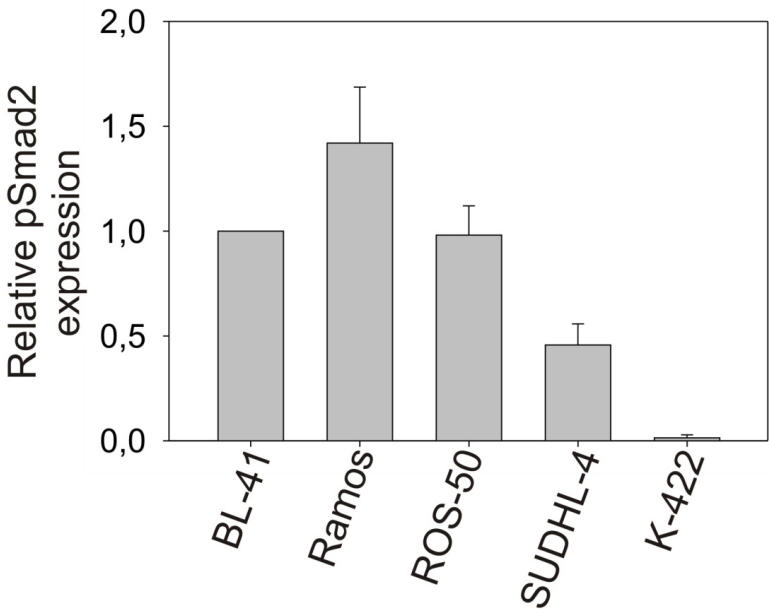
Additional file 1, Fig. S1.



Additional file 2, Fig. S2.



Additional file 3, Fig. S3.



Additional file 4, Fig. S4.

